EXHIBIT A

AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B

Catherine S. Manno, Amy J. Chew, Sylvia Hutchison, Peter J. Larson, Roland W. Herzog, Valder R. Arruda, Shing Jen Tai, Margaret V. Ragni, Arthur Thompson, Margareth Ozelo, Linda B. Couto, Debra G. B. Leonard, Frederick A. Johnson, Alan McClelland, Ciaran Scallan, Erik Skarsgard, Alan W. Flake, Mark A. Kay, Katherine A. High, and Bertil Glader

Hemophilia B is an X-linked coagulopathy caused by absence of functional coagulation factor IX (F.IX). Previously, we established an experimental basis for gene transfer as a method of treating the disease in mice and hemophilic dogs through intramuscular injection of a recombinant adeno-associated viral (rAAV) vector expressing F.IX. In this study we investigated the safety of this approach in patients with hemophilia B. In an open-label dose-escalation study, adult men with severe hemophilia B (F.IX < 1%) due to a missense mutation were injected at multiple intramuscular sites with an rAAV vector. At doses ranging from 2×10^{11} vector genomes (vg)/kg to 1.8×10^{12} vg/kg, there was no evidence of local or systemic toxicity up to 40 months after injection. Muscle biopsies of injection sites performed 2 to 10 months after vector administration confirmed gene transfer as evidenced by Southern blot and transgene expression as evidenced by immunohistochemical staining. Preexisting high-titer antibodies to AAV did not prevent gene transfer or expression. Despite strong evidence for gene transfer and expression, circulating levels of F.IX were in all cases less than 2% and most were less than 1%. Although more extensive transduction of muscle fibers will be required to develop a therapy that reliably raises circulating levels to more than 1% in all subjects, these results of the first parenteral administration of rAAV demonstrate that administration of AAV vector by the intramuscular route is safe at the doses tested and effects gene transfer and expression in humans in a manner similar to that seen in animals. (Blood. 2003;101:2963-2972)

Introduction

Hemophilia B is an X-linked bleeding disorder caused by mutations in the gene encoding blood coagulation factor IX (F.IX), a zymogen of a serine protease required for thrombin generation. The clinical severity of hemophilia correlates with circulating F.IX levels. Patients with less than 1% circulating F.IX typically experience spontaneous hemorrhages and prolonged bleeding after trauma or surgery. Treatment requires intravenous infusion of clotting factor concentrates that are either derived from plasma or made through recombinant technology. Despite recent improvements in the safety profiles of factor concentrates, morbidity and mortality persist.[1] The major morbidity is arthropathy, resulting from recurrent bleeding into joint spaces. Mortality can result from bleeding into critical closed spaces (eg, intracranial or intraperitoneal bleeding),[2-5] although the leading causes of death currently in the US hemophilia population (HIV and hepatitis) are a consequence of transfusion-transmitted infection from early-generation plasma-derived concentrates.[6]

Based on the natural history of disease in patients with baseline factor levels more than 1% and on studies of hemophilia patients treated with routine administration of clotting factor concentrates to maintain levels more than 1%, it is likely that sustained expression of clotting factor at levels more than 1% could prevent serious bleeding complications and preserve joint function.[7] An advantage of hemophilia as a model for gene transfer is that tissue-specific expression of the transgene is not required, because biologically active F.IX can be produced in cells other than hepatocytes.[8,9] In addition, precise regulation of transgene expression is not required, because levels of 1% to 2% may be therapeutic and levels up to 100% are still within the normal range. The existence of small and large animal models of this disease[10-16] facilitates analysis of efficacy before clinical studies are initiated, and measurement of clinical therapeutic end points (circulating levels of F.IX) is straightforward.

Adeno-associated viral (AAV) vectors transduce a variety of somatic tissues including liver, central nervous system, and skeletal muscle.[17-21] The preclinical experiments that led to this clinical trial established that intramuscular administration of an AAV vector encoding F.IX resulted in long-term expression in mice and in

From the Departments of Pediatrics, Surgery, and Pathology, University of Pennsylvania, Philadelphia; Children's Hospital of Philadelphia, PA; Avigen, Inc, Alameda, CA; Departments of Pediatrics and Surgery, Stanford University School of Medicine, Stanford, CA; Bayer Corporation Biological Products Division, Research Triangle Park, NC; Hemophilia Center of Pennsylvania, University of Pittsburgh, PA; Puget Sound Blood Center, Seattle, WA; and Hematology-Hemotherapy Center, State University of Campinas, Campinas-SP, Brazil.

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Reprints: Catherine S. Manno, Children's Hospital of Philadelphia, 34th St and Civic Center Blvd, Division of Hematology, 4 Wood, Philadelphia, PA 19104; e-mail: manno@email.chop.edu.

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Table 1. Dose escalation* and revision

Group/no. patients	Dose/site, vg†	Dose/kg, vg‡	Total dose, vg§	Legs injected
Original outline for dose escalation				
1/3	2.3×10^{12}	2.0×10^{11}	1.4×10^{13}	One or both
2/3	7.0×10^{12}	2.0×10^{12}	1.4×10^{14}	Both
3/3	3.5×10^{13}	1.0×10^{13}	7.0×10^{14}	Both
Revised plan used in clinical study				
1/3	1.5×10^{12}	2.0×10^{11}	1.4×10^{13}	One or both
2/3	1.5×10^{12}	6.0×10^{11}	4.2×10^{14}	Both
2/2	1.5×10^{12}	1.8×10^{12}	1.3×10^{14}	Both‖

*After the death of a patient in a gene transfer trial, the sponsors and investigators in this trial voluntarily slowed the pace of dose escalation from a one-log increase between the low and mid-dose cohorts to 1/2 log between each dose cohort.

†Vector genomes.

‡Dosing performed according to the patient weight obtained at the time of injection

§Assuming a 70-kg adult

‖Plus auxiliary muscles (deltoid, soleus).

hemophilic dogs.[22,23] We previously reported initial evidence of gene transfer in the first 3 human subjects receiving parenteral injections of an AAV vector.[24] We now report complete results of this phase 1 safety study, the first in which an AAV vector was used for gene transfer in hemophilia.

Patients, materials, and methods

Vector

The AAV-human F.IX (AAV-hF.IX) vector is derived from AAV serotype 2 using recombinant DNA techniques and contains a F.IX minigene expression cassette of 4071 nucleotides between the 2 viral inverted terminal repeats (ITRs). The F.IX expression cassette contains: (1) a cytomegalovirus (CMV) enhancer/promoter fragment[22]; (2) exon 1 of the human F.IX (*F9*) gene; (3) a portion of the human F.IX intron I[25]; (4) exons 2-8 of the human *F9* gene; and (5) the SV40 late polyadenylation sequence. Vector was manufactured under good manufacturing practice (GMP) conditions using a triple transfection procedure in 293 cells as previously described.[26] Vector was titered by quantitative DNA dot-blot. Final product testing prior to lot release included a F.IX potency assay and assays for sterility and endotoxin as previously described.[27] The study was conducted using 4 separate lots of vector.

Subjects

Eight subjects were enrolled, 3 in both the low- and medium-dose cohorts and 2 in the high-dose cohort. Enrollment of a subject did not proceed until the previous subject was observed for at least 2 weeks; enrollment in a higher dose cohort continued only after the previous cohort had been observed for at least 4 weeks. The original and revised dose-escalation plans are outlined in Table 1.

Subjects were recruited from hemophilia treatment centers in North and South America. Prior to subject enrollment, the clinical protocol was reviewed and approved by the US Food and Drug Administration, the National Institutes of Health Recombinant DNA Advisory Committee of the Office of Biotechnology Activities, General Clinical Research Centers (GCRC) of the Children's Hospital of Philadelphia, Stanford University Medical Center, local institutional review boards, and institutional biosafety committees. Subjects gave written informed consent prior to treatment with AAV-F.IX. We initially preferred and ultimately required that only subjects with a missense mutation be included. The rest of the inclusion and exclusion criteria are listed in Table 2. Infection with HIV or hepatitis C virus (HCV) did not preclude participation.

Procedure

Prior to administration of the vector, subjects received 100% correction with F.IX concentrate. Analgesia included either local anesthesia, conscious sedation, or general anesthesia based on patient preference. For subjects in group 1, vector was administered into one or both vastus lateralis muscles. This muscle group was chosen because it is easily inspected and palpated. The volume injected at each site did not exceed 500 μL.

Dose of vector

Based on animal data suggesting that the risk of inhibitor formation was influenced by the dose administered per injection site,[28] we limited the dose to 1.5×10^{12} vector genomes (vg)/site. Injections were administered under ultrasound guidance to minimize the risk of injection into a large blood vessel. Injections were spaced at least 1 cm apart. The number of injection sites for subjects in the low-dose cohort was between 10 and 20, and for the mid-dose cohort 30 to 50. For the high-dose cohort, additional skeletal muscles, including the deltoid and the soleus, were used for injections, and a total of 80 to 90 injections was made. Several injection sites were marked with a small intradermal injection of India ink intended to aid in identifying sites for muscle biopsies to be performed months after injection.

Muscle biopsy

Muscle biopsies were planned at 2, 6, and 12 months after vector injection. Muscle tissue obtained by biopsy was immediately frozen in liquid nitrogen–cooled isopentane and stored at −80°C prior to preparation of cryosections.[22,24] Sections were stained with hematoxylin and eosin to evaluate histology. Immunoperoxidase staining of cryosections for F.IX expression was carried out as described using a goat antihuman F.IX (Affinity Biologicals, Hamilton, ON, Canada) as primary antibody at a dilution of 1:400.[24] Similarly, this antibody was also used for immunofluorescence staining of F.IX expressed in muscle sections using the previously published protocol.[22] The secondary antibody in this assay was fluorescein isothiocyanate (FITC)–conjugated rabbit antigoat IgG (Dako, Carpinteria,

Table 2. Eligibility criteria

Inclusion criteria
Males with severe F.IX deficiency
Age 18 y or older
Ability to give informed consent
More than 20 exposure days of treatment with F.IX protein
No history or presence of an inhibitor to F.IX protein
Able to infuse F.IX protein on a home infusion protocol
Subjects with F.IX missense mutations
Exclusion criteria
Active infections
End-stage renal disease
Severe liver disease defined as any of the following:
Bilirubin; 2.1-3.0 × normal
Transaminases: 5-10 × normal
Alkaline phosphatase: 5-10 × normal
Platelet count less than 50 000/μL
Presence of inflammatory muscle disease (eg, myositis)

Table 3. Subject demographics

	A*	B*	C*	D	E	F	G	H
Age, y	38	23	67	29	44	43	38	30
Race	White	Asian	White	White	Asian	White	White	White
Baseline F.IX	<1%	<1%	<1%	<1%	<1%	<1%	<1%	<1%
CRM status	+	−	−	+	+	−	+	+

CRM indicates cross-reacting material.
*Included in report by Kay et al.[24]

CA) as described.[22] For staining of slow-twitch muscle fibers, cryosections were allowed to thaw at room temperature (without fixation), blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) for 1 hour, and subsequently incubated at 4°C overnight using the monoclonal NCL-MHCs (Novacastra Laboratories–myosin heavy chain) antibody (Novacastra Laboratories, Newcastle upon Tyne, United Kingdom; 1:20 in PBS/1%BSA) specific for slow-twitch myosin. After three 10-minute washes at room temperature in PBS, sections were incubated with tetrarhodamine isothiocyanate (TRITC)–labeled goat antimouse IgG (Sigma, St Louis, MO) for 2 hours at room temperature followed by 3 additional PBS washes. Serial sections of muscle tissue were fluorescence stained for F.IX or slow-twitch myosin and compared for expression of either antigen using a Nikon fluorescent microscope. For double staining, antislow myosin was applied simultaneously with antiheparan sulfate proteoglycan (HSPG; 1:100; Chemicon, Temecula, CA). In another experiment, muscle sections were prepared and stained with monoclonal antibody NCL-MHCs or NCL-MHCf (specific for slow- or fast-twitch myosin, respectively) as described followed by staining with a peroxidase-conjugated secondary antibody. Peroxidase-stained slides were examined by light microscopy.

Laboratory evaluation

Routine clinical laboratory testing was performed using procedures approved by the College of American Pathology (CAP) for serum chemistries, hematologic values, coagulation factor assays, and Bethesda assays. Specifically, F.IX activity levels were determined using an automated analyzer (MDA, Bio-Mérieux, Research Triangle Park, NC; or MLA-800, Medical Laboratory Automation, Pleasantville, NY). Plasma test samples were mixed with F.IX-deficient substrate (George King Biomedical, Overland Park, KS) and results were compared with the degree of correction obtained when dilutions of known reference plasma were added to the same F.IX-deficient substrate. The reference curve was linear down to a lower limit of 0.3%. Bethesda assays were carried out using a standard procedure in which residual F.IX activity is determined after incubating equal volumes of test plasma with normal pooled plasma at 37°C for 2 hours. The lower limit of detection in this assay is 0.1 Bethesda units (BU). In addition to these CAP-approved procedures, Western blotting was done to detect anti-F.IX antibodies, as previously described.[24] Positive controls included serum samples from a patient with a history of F.IX inhibitory antibody (Bethesda titer 24 BU). We used a polymerase chain reaction (PCR) assay to detect vector sequences in body fluids (serum, urine, saliva, semen, and stool) and in skeletal muscle. The 5′ primer was derived from the CMV enhancer/promoter (5′-AGTCATCGCTATTACCATGG-3′) and the 3′ primer from intron 1 of the human F.IX (*F9*) gene (5′-GATTTCAAAGTGGTAAGTCC-3′). Amplified vector sequence yields a PCR fragment of 743 bp. For each sample, a control reaction containing the sample to be tested spiked with vector plasmid (50 copies/μg DNA) was also run to establish that the sample did not inhibit the PCR reaction. For semen, 3 μg DNA was analyzed (1 μg in each of 3 separate reactions); for saliva and biopsied muscle, 1 μg; and for urine, serum, and stool, DNA was extracted from a 1- to 2-mL volume and analyzed. The sensitivity of the assay is 50 copies of vector sequence in 1 μg DNA. AAV-neutralizing antibodies were measured by incubating an AAV vector expressing lacZ for 60 minutes with serial dilutions of patient serum, then using this mixture to transduce HEK293 cells. Cells were lysed 24 hours after transduction and β-galactosidase activity was determined by enzymatic assay; sera or dilutions were scored as positive for neutralizing AAV antibodies if the OD_{420} was 50% or less that observed when rAAV-lacZ was preincubated with negative control mouse sera.

DNA analysis

Total gDNA was isolated from frozen muscle tissue using the PureGene kit from Gentra Systems (Minneapolis, MN). Vector sequences were detected using Southern blot hybridization[29] or PCR (see "Laboratory evaluation"). For Southern hybridization, a vector-specific 0.7-kb ^{32}P-labeled *Bgl*II-fragment including the CMV enhancer/promoter sequence was used as a probe and gene copy number was determined by comparison with controls spiked with known amounts of plasmid DNA. The intensities of bands on autoradiographs were quantitated by densitometric scanning.

Results

We enrolled 8 adult men with severe hemophilia B (Table 3). F.IX mutation analyses, a prerequisite for study entry, demonstrated underlying missense mutations in all subjects enrolled (Table 4).

Two subjects (A and D) were HIV$^+$, and subject A was on highly active antiretroviral therapy at the time of vector injection with a CD4 count of more than 300/μL and undetectable HIV viral load. CD4 counts in subject D ranged from 597 to 864/μL during the course of the study; he was taking no antiretroviral medications at that time. Seven of 8 were previously infected with HCV as detected by presence of antibody to HCV. No subjects were receiving interferon or ribavirin at the time of treatment with AAV-hF.IX (Table 5).

Clinical observations and laboratory studies

Intramuscular injection of vector doses up to 1.8×10^{12} vg/kg was well-tolerated in all subjects, with no systemic symptoms or signs noted during the 24 hours of hospitalization immediately following vector administration and none observed in the ensuing period of close outpatient follow-up.

Laboratory studies revealed no abnormalities in serum chemistries, save for in one subject a 5-fold elevation in the creatine phosphokinase (CPK), which returned to baseline 1 week after injection. Complete blood counts also demonstrated no abnormalities, except for patient F. This subject, with a history of thrombocytopenia secondary to liver disease, had a platelet count of 111 000/μL 3 days after vector injection (not lower than previous values in this subject); the platelet count returned to the subject's pretreatment baseline of 140 000/μL 5 days later.

Table 4. Mutation analysis of subjects

A*	B*	C*	D	E	F	G	H
Arg4Leu	Ala352Pro	Gly114Arg	Cys18Arg	Gly184Arg	Ser110Pro	Arg180Trp	Pro368Thr
Nucl no. 6365	Nucl no. 31172	Nucl no. 17755	Nucl no. 6427	Nucl no. 20529	Nucl no. 17743	Nucl no. 20492	Nucl no. 31223
CGG>CTG	GCT>CCT	GGA>CGA	TGT>CGT	GGA>AGA	TCC>CCC	CGG>TGG	CCC>ACC

*See Kay et al.[24]

Table 5. Infection status of subjects

	A*	B*	C*	D	E	F	G	H
HIV	+	−	−	+	−	−	−	−
HCV	+	+	−	+	+	+	+	+
HBV	−	−	+	+	−	−	−	−
HAV	−	−	−	−	−	−	−	−

+ indicates positive; −, negative; HBV, hepatitis B virus; and HAV, hepatitis A virus.

*See Kay et al.[24]

Adverse events

Other than the above episode of thrombocytopenia, there were no vector-related toxicities in the low-, medium-, or high-dose cohorts. Four of 8 subjects developed transient minor abnormalities (hematoma, induration, transient numbness) at the site of muscle biopsy. Five of 8 subjects developed small hematomas or pain at one or more vector injection sites, which resolved uneventfully. Finally, one subject had a mild inflammatory reaction to the tattoo dye that was injected at a few sites; these resolved without treatment.

Safety studies

In biodistribution studies based on a sensitive PCR assay, vector DNA was detected in the serum of all subjects at 24 hours, and up to but not after day 7 in all except subject E in whom vector sequences were detected up to 12 weeks after injection. Vector sequences were detected in the urine of subjects B, D, and F up to 24 hours after injection but not thereafter. Saliva was positive for vector sequences in 7 of 8 subjects as early as 24 hours and as late as 14 days in subject G. Vector sequences were not detected at any time point in the semen of any of the 7 subjects tested (Table 6). One subject was unable to provide semen due to erectile dysfunction. None of the subjects had had a vasectomy.

No inhibitory antibodies to F.IX were detected by Bethesda assay during the period of follow-up, despite repeated challenges with intravenous infusion of F.IX concentrates. In addition, noninhibitory antibodies to F.IX were sought using Western blotting, and also were not detected at any of the time points tested in the subjects in this study (Figure 1). Neutralizing antibodies to AAV were detected prior to treatment in 7 of 8 subjects. Anti-AAV antibody titers rose in all subjects following vector injection, demonstrating an intact immune response, even in subjects who were HIV+ (Table 7). Analysis of muscle biopsies (see "Gene transfer and expression") shows no correlation between pretreatment titer of anti-AAV antibodies and evidence for gene transfer and expression on muscle biopsy.

Gene transfer and expression

Direct evidence for gene transfer and expression was sought on muscle biopsies obtained 2 months (8 subjects), 6 months (1 subject), and

Table 6. PCR analysis of body fluids for vector sequences

			Days after injection								Weeks after injection											
Sample	Subject	Baseline	0	1	2	3	4	5	6	7	2	3	4	5	6	7	8	10	12	14	16	24
Serum	A*	−	ND	+	+	ND	−	ND	ND	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−	−	ND
	B*	−	+	+	−	ND	ND	ND	ND	−	−	−	−	ND	−	ND	−	−	ND	ND	ND	ND
	C*	−	−	+	+	ND	ND	ND	−	−	ND	−	−	ND	−	ND	−	ND	ND	ND	ND	ND
	D	−	−	+	+	ND	ND	ND	ND	−	ND	−	−	−	−	−	−	ND	ND	ND	ND	ND
	E	−	ND	ND	+	ND	ND	ND	ND	+	−	ND	+	ND	ND	+	−	−	+	ND	ND	ND
	F	−	ND	+	ND	ND	ND	ND	ND	+	ND	ND	−	ND	−	ND	−	ND	−	ND	ND	ND
	G	−	−	+	+	ND	ND	ND	ND	+	−	−	ND	ND	ND	ND	ND	ND	−	ND	ND	ND
	H	−	ND	ND	ND	ND	+	ND	ND	+	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Saliva	A*	−	−	+	−	ND	ND	ND	ND	−	ND	ND	−	ND	ND	−	−	ND	ND	ND	ND	ND
	B*	−	ND	+	−	−	ND	ND	ND	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND
	C*	−	−	+	−	ND	ND	ND	ND	−	ND	ND	−	ND	−	ND	ND	ND	ND	ND	ND	ND
	D	−	+	+	−	ND	ND	ND	ND	ND	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	E	−	ND	ND	+	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	−	−	−	−	ND	ND	ND
	F	−	ND	+	ND	ND	ND	ND	ND	−	ND	ND	−	ND	−	ND	−	ND	ND	ND	ND	ND
	G	−	fp	fp	+	ND	ND	ND	ND	−	+	−	ND	ND	ND	ND	ND	ND	−	ND	ND	ND
	H	−	ND	ND	ND	−	−	ND	ND	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Urine	A*	−	−	−	−	ND	ND	ND	ND	ND	−	ND	−	ND	ND	−	−	ND	ND	ND	ND	ND
	B*	−	ND	+	−	ND	ND	ND	ND	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND
	C*	−	ND	−	−	ND	ND	ND	−	−	ND	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
	D	−	−	−	−	ND	ND	ND	ND	−	−	−	ND	ND	ND	ND	−	ND	ND	ND	ND	ND
	E	−	ND	−	ND	ND	ND	ND	ND	−	−	−	−	ND	ND	−	−	−	−	ND	ND	ND
	F	−	+	ND	ND	ND	ND	ND	ND	−	ND	ND	−	ND	−	ND	−	ND	−	ND	ND	ND
	G	−	−	−	−	ND	ND	ND	ND	−	−	−	−	ND	ND	ND	ND	ND	−	ND	ND	ND
	H	−	ND	ND	ND	−	−	ND	ND	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Semen	A*	−	ND	ND	−	−	ND	ND	ND	−	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	ND
	B*	−	ND	ND	ND	ND	−	ND	ND	−	ND	ND	−	−	−	−	ND	ND	ND	ND	ND	ND
	C*	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND
	D	−	ND	ND	ND	−	ND	ND	ND	ND	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	E	−	ND	ND	ND	ND	ND	ND	ND	−	ND	ND	−	ND	ND	ND	−	ND	ND	ND	ND	ND
	F	−	ND	ND	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	−
	H	fp	ND	ND	ND	ND	−	ND	ND	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

No semen samples were taken from subject G due to erectile dysfunction secondary to sertraline. Semen analysis in subject H was faintly positive with nonspecific bands.
+ indicates positive; −, negative; fp, faint positive; and ND, not done.

*See Kay et al.[24]

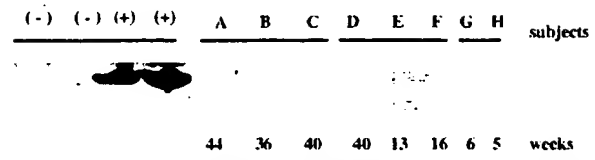

Figure 1. Western blot for detection of antibodies to human F.IX in subjects in study. Serum samples were collected at monthly intervals from all subjects and used in a 1:1000 dilution as the primary antibody in an immunoblot to detect antibodies to human F.IX. Shown here are samples from each subject, drawn at a series of time points ranging from 5 weeks to 44 weeks after vector injection. The positive control (+) is performed using serum from a patient with an inhibitor (24 BU) to F.IX and the negative control is serum from a healthy subject.

10 months (1 subject) after vector administration. Of 10 biopsies performed, 8 of the 10 were positive for the donated gene on PCR assay, and 5 of 9 analyzed were positive on Southern blot (Table 8). Southern blot, though less sensitive, is more informative than the simple PCR assay because it allows assessment of molecular form and copy number of vector DNA in the sample. Southern blot analyses containing DNA from 6 subjects are shown in Figure 2. Vector DNA is detected in the undigested sample as a high-molecular-weight smear (Figure 2B, lane 7; Figure 2C, lane 6; Figure 2D, lanes 4 and 10). Whether the vector DNA is integrated or is stabilized in a high-molecular-weight episomal form is not clear from this analysis. Following digestion with *Eco*RI, which cleaves once in the minigene cassette, vector signal is detected as a 4.5-kb fragment (size of the vector insert, Figure 2B, lanes 5 and 10; Figure 2C, lane 7; Figure 2D, lane 6). Release of a unit length vector fragment indicates formation of circular forms or concatemers of the vector genome. Digestion with *Bam*HI (which also cuts once in the vector; Figure 2A) revealed the presence of both head-to-tail and head-to-head arrangements (Figure 2B, lane 8; Figure 2C, lane 8; Figure 2D, lanes 7 and 12). In lanes with undigested DNA, a faint band migrating somewhat lower than the 4.5-kb *Eco*RI fragment was observed, and likely represents a monomeric circular form of the vector genome (Figure 2B, lane 7; Figure 2D, lane 10). Digestion with *Bgl*II releases a 0.7-kb fragment containing the CMV promoter/enhancer (Figure 2B, lanes 4 and 9; Figure 2C, lane 5; Figure 2D, lanes 5 and 11). The *Bgl*II digests were used to estimate gene copy number in the sample, as judged against a series of standards (Figure 2B-D, lanes 1-3, respectively). For lanes with a positive signal, the gene copy number was generally in the range of 0.5 to 4 copies/human diploid genome (Table 8). Gene transfer could be demonstrated on biopsy samples taken as late as month 10 after vector administration (Figure 2C, lanes 5-8). Examples of undetectable gene transfer by Southern blot (< 0.5 copies/diploid genome) are shown in lane 4 of Figure 2C and lanes 8 and 9 in Figure 2D (summarized in Table 8).

Table 7. Neutralizing AAV antibody titers

	Baseline	~1 mo	~6 mo
A	1:100*	1:5000*	1:1000
B	1:1000*	1:10 000*	1:10 000
C	1:10*	1:10 000*	1:1000
D	1:100	1:10 000	1:10 000
E	1:100	1:1000	1:1000
F	1	1:1000	1:1000
G	1:100	1:10 000	1:10 000
H	NS	NS	1:1000

293 cells were incubated with serial dilutions of patient serum and transduced with AAV lacZ. Sera scored positive for neutralizing AAV antibodies if β-galactosidase activity is 50% or less of that observed when rAAV-lacZ was preincubated with negative control mouse sera.

NS indicates no sample.

*See Kay et al.[24]

Table 8. Bioactivity and efficacy studies in subjects treated with intramuscular AAV-hFIX

	PCR on muscle biopsy	Southern blot on muscle biopsy*	F.IX immunohistochemistry	Max circ of F.IX	Decrease in F.IX infusion
A	Post†	Neg	Neg	1.40%	50%
B	Post†	ND	Pos	<1%	50%
C	Post†	Pos (4)	Neg	<1%	None
D	Pos	Pos (1.5)	Pos	<1%	None
	Pos	Pos (2.5)	Pos	<1%	None
E	Neg	Neg	Pos	<1%	None
F	Neg	Neg	Pos	1%	None
	Pos	Pos (0.5)	Pos	1%	None
G	Pos	Neg	Pos	<1%	None
H	Pos	Pos (0.5)	Pos	<1%	None

Max circ indicates maximum circulation of F.IX; ND, study not performed because of insufficient tissue.

*Gene copy number estimates, based on comparison to standards, are shown in parentheses.

†See Kay et al.[24]

To assess expression of the donated gene in biopsied muscle, we performed both immunofluorescent and immunoperoxidase staining for F.IX (Figure 3). All examined tissue samples showed healthy muscle architecture without evidence for inflammation (Figure 3A and data not shown). Eight of 10 biopsies contained areas positive for expression of the donated gene up to month 10 after transduction (Table 8); these were typically found adjacent to tissue blocks negative for transgene expression, likely dependent on how close the biopsy samples were located to the main site of injection. All positive samples examined showed a mosaic-like pattern of F.IX staining, with brightly staining positive fibers directly adjacent to negative fibers (Figure 3B-G). Furthermore, there was extensive extracellular staining of the secreted F.IX (Figure 3B,D-G). This pattern is identical to that seen previously in injected animal tissues.[22,23] In a few cases (eg, subjects A, E, and F) there was discordance between results for gene transfer and expression. This likely proceeds from the exigencies of processing a small sample for multiple studies: each muscle biopsy sample was subdivided into fragments that were then used independently for PCR, Southern blot, or immunohistochemistry. Based on previous experience with muscle biopsies in large animals, it is clear that sampling of injected sites can be an imprecise process and that biopsy material can routinely contain injected tissue as well as uninjected adjacent tissue.

In addition to the studies performed to assess gene transfer and expression, we performed other immunohistochemical studies in an attempt to elucidate the basis of the mosaic-like pattern of transgene positivity seen in the muscle samples. Based on a report by Huard and colleagues,[30] we hypothesized that slow fibers of human muscle are preferentially transduced by rAAV-2. Pruchnic et al showed abundant presence of HSPGs, which act as a receptor for AAV-2,[31] in murine slow-twitch (but not fast-twitch) fibers. Adjacent sections from the muscle biopsy of subject G were stained with antibodies to slow-twitch myosin or to human F.IX (Figure 4E-F). These showed excellent concordance between slow-twitch fibers and F.IX expression. In another experiment we found excellent concordance between expression of HSPGs and of slow-twitch myosin in muscle fibers (Figure 4C-D). Finally, the percentage of muscle fibers in the vastus lateralis muscle was determined to be approximately 30% to 40% slow-twitch and 60% to 70% fast-twitch fibers (Figure 4A-B). This is in good agreement with older literature assessing slow/fast-twitch fiber composition for this muscle in humans.[32]

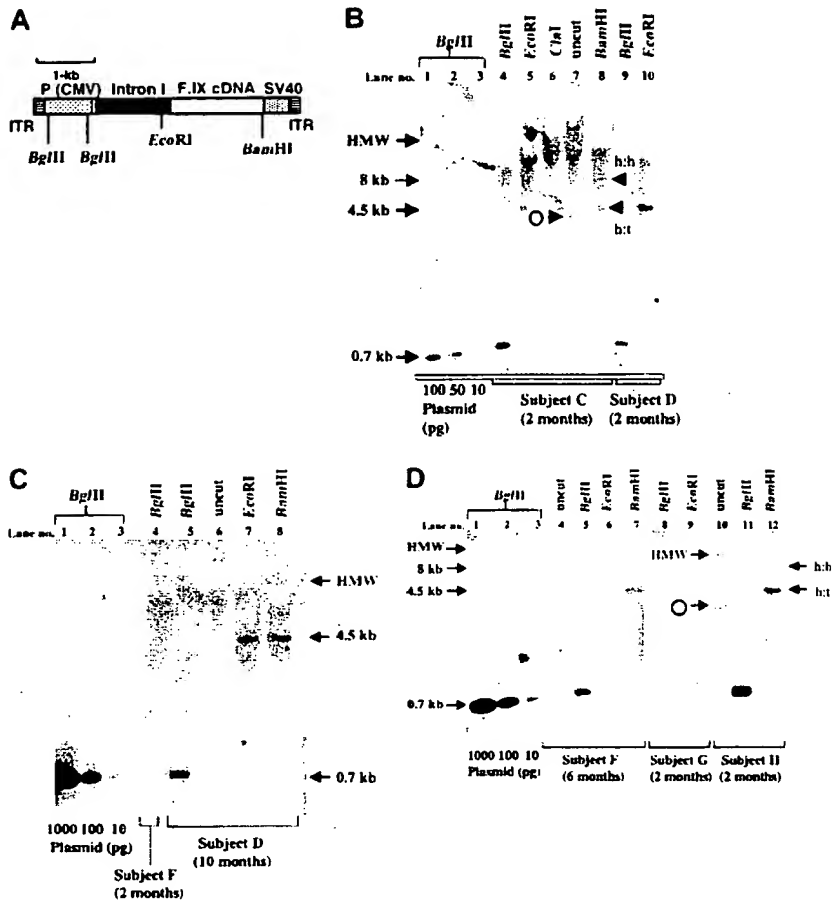

Figure 2. Southern blot analysis of gDNA isolated from injected human skeletal muscle tissue. (A) Diagram of AAV vector containing the CMV IE enhancer/promoter, P (CMV), exon 1 and a 1.4-kb portion of intron 1 the human F.IX (*F9*) gene (intron I), exons 2-8 of the human F.IX cDNA (F.IX cDNA) including 0.2-kb of the 3′-untranslated region, and the SV40 polyadenylation signal (SV40). The expression cassette is flanked by AAV-2 ITRs. (B-D) Total gDNA was isolated from biopsied muscle tissue and restricted with *Bgl*II to release a vector-specific 0.7-kb fragment (CMV IE enhancer/promoter), or restricted with *Eco*RI, which cuts once in the middle of the vector resulting in a 4.5-kb fragment (unit length of the vector) for vector sequences present as concatemers or monomeric circles. Alternatively, gDNA was restricted with *Cla*I (which does not cut in the vector genome) or with *Bam*HI, which cuts once within the vector and thus allowing a distinction between head-to-tail (4.5-kb) and head-to-head/tail-to-tail (8-kb and 1-kb, respectively) concatemeric arrangement. Plasmid standards (10-1000 pg/lane) encoding the AAV vector genome were cut with *Bgl*II for estimation of gene copy number. gDNA (15 μg, restricted or undigested) and pDNA were separated on 1% agarose gels, Southern blotted onto a nylon membrane, and probed with a ^{32}P-random prime-labeled 0.7-kb *Bgl*II fragment representing the CMV enhancer/promoter. Sizes of bands were estimated by comparison with a size marker (1-kb ladder; Gibco BRL). Indicated are high-molecular-weight (HMW), putative head-to-head (h:h) and head-to-tail (h:t) fragments (note that a tail-to-tail fragment is not recognized by the probe) and circular monomeric forms (°). Southern blot analyses are shown for muscle biopsy of subjects C and D (panel B, 2 months after vector administration), subjects D and F (panel C, 10 and 2 months after vector administration, respectively), and subjects F, G, and H (panel D, 6, 2, and 2 months after vector administration, respectively).

Due to the morbidity of the procedure, subjects were reluctant to proceed with subsequent muscle biopsies, and only 2 biopsies were available from the 6-month and 10-month time points. Both showed evidence for gene transfer and expression, with copy number and expression undiminished compared to the 2-month time points.

Factor usage

Prior to vector injection, usage of factor concentrate in subject A, administered in an on-demand regimen, averaged 3.7 treatments per week. During 2 years of follow-up after vector administration, he reduced factor usage by 50%.

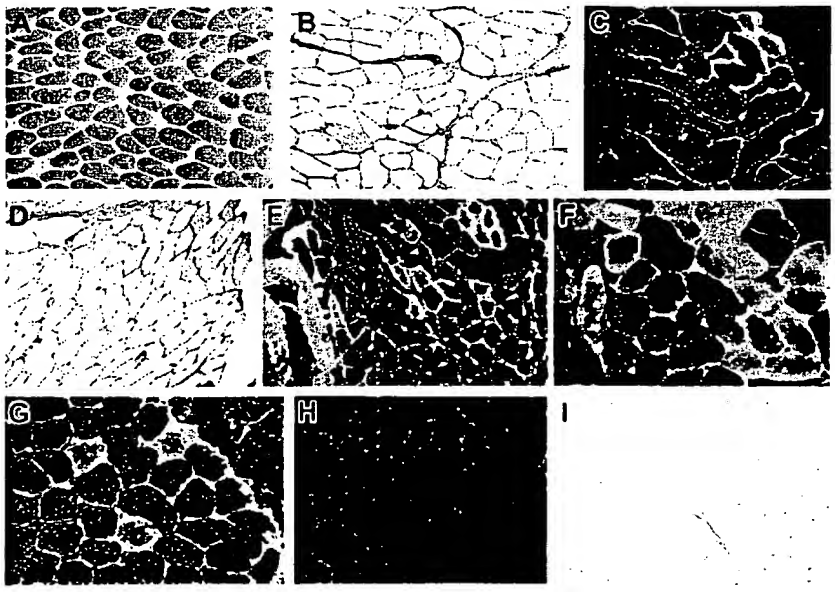

Figure 3. Histology of skeletal muscle cross-sections of biopsy taken 2 months after vector administration. (A-C) Subject D, hematoxylin and eosin (A), F.IX immunohistochemistry (B, brown stain), and F.IX immunoflourescence stain (C, green stain). (D-G) Subject G F.IX immunohistochemistry (D, brown stain), and F.IX immunofluorescence stain (E-G, green stain). (H-I) Sections that stained negative for F.IX expression by immunohistochemistry (H) and immunofluorescence (I) methods for comparison. Original magnifications × 100 (A-B, D-E, H-I) and × 200 (C, F-G).

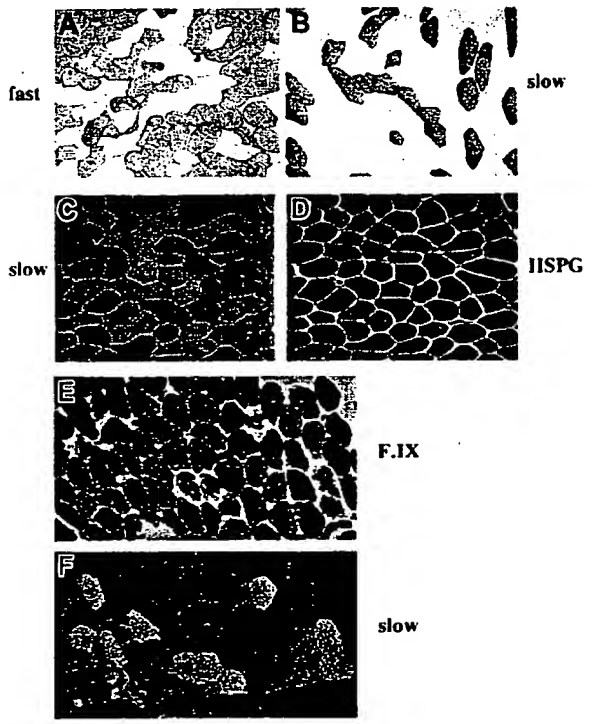

Figure 4. Histochemical analysis of human vastus lateralis muscle and vector transduction. (A-B) Immunohistochemical stain for fast-twitch (A) and slow-twitch (B) isoforms of myosin. (C-D) Simultaneous immunofluorescence stain for slow-twitch myosin (C) and HSPG (D) indicates cytoplasmic HSPG stain in slow-twitch muscle fibers. (E-F) Colocalization of F.IX transgene expression (E) and slow-twitch muscle fibers (F) on serial sections of biopsy from subject G, 2 months after vector administration. Original magnification × 100 for panels A-F.

Subject B treated himself on average 4.1 times a week prior to treatment with the vector. During the first 24 months after vector treatments (and continuing to the present), he reduced his factor concentrate usage by 50%. There were no changes in the treatment patterns recorded by subjects C through H before and after vector administration.

Factor levels

Four of the 8 subjects had F.IX levels higher than baseline at time points that were at least 10 days after the most recent factor infusion. Subject A was first noted to have a F.IX level higher than baseline at 8 weeks after injection and was again noted to have a level higher than baseline (1.4%) at 12 weeks, 14 days after the most recent factor infusion. This sample was measured at more than 1% in 4 different clinical laboratories. Subject A had a 1% level measured 52 weeks after treatment with the vector. Subject B also showed measurable increases in F.IX levels, from a baseline of less than 0.3%, up to 0.8%. Subject D had a measured level of 0.7% at 8 weeks, with a baseline of 0.2%. Subject G had a level of 0.8% at 4 weeks after treatment. All other F.IX levels, measured at least 14 days after factor concentrate infusion, were less than 1% in the remaining subjects (Table 9).

Discussion

This report describes the first clinical study in which an AAV vector was administered by parenteral injection. Recombinant AAV in doses up to 1.8×10^{12} vg/kg was well tolerated when introduced into skeletal muscle, and there was no evidence of serious local or systemic toxicity. Muscle biopsies demonstrated gene transfer and expression in the majority of subjects tested, with one biopsy (the only one performed at a late time point) documenting undiminished gene copy number and expression 10 months after vector administration. This observation is consistent with preclinical studies that have documented expression in mice for over 1 year after intramuscular injection and in dogs more than 4 years after injection[22,23] (R.W.H. et al, unpublished results, 2001).

The design of this initial trial reflects safety considerations arising from the lack of clinical experience with rAAV and from characteristics unique to the hemophilia population. In preclinical studies, we demonstrated that either skeletal muscle or liver could serve as a target tissue for AAV-mediated gene transfer and that therapeutic levels of circulating F.IX could be obtained in mice and hemophilic dogs with either route of administration.[21-23,33,34] Several factors influenced the decision to use skeletal muscle as the target in these initial clinical studies. At the time that the study was begun (June 1999), there was no experience with parenteral administration of AAV; the only prior human studies had been (topical) administration of rAAV into the maxillary sinuses or the respiratory tracts of individuals with cystic fibrosis.[35] Thus, parenteral studies were initiated at a peripheral rather than systemic site. In addition, intramuscular injection is a familiar and relatively noninvasive technique, whereas administration of vector to the hepatic circulation requires an interventional radiology procedure at a minimum. This also favored muscle as a target tissue. The high prevalence of hepatitis in the adult hemophilia population[36-38] also dampened enthusiasm for a liver-directed approach. Finally, biodistribution studies in mice and rabbits suggested that the risk of inadvertent germline transmission of vector sequences was lower with an intramuscular approach,[39-42] a supposition since supported by findings in the first subjects enrolled in a subsequent liver-directed trial.[43-45] On the other hand, it was clear from our own studies and those of others that there was a dose advantage in favor of liver,[21,34,46-48] and that all the necessary posttranslational modifications would be accurately and efficiently executed in the hepatocyte, a condition that does not always obtain in skeletal muscle.[8]

Table 9. F.IX assay percentages

	A	B	C	D	E	F	G	H
2 wk	14*	13*	1.4*	0.7	0.4	0.7	0.5	0.5
4 wk	3*	<1	<1	0.2	1.7*	0.3	0.8	0.3
8 wk	1*	<1	0.6	0.7	0.3	0.2	<1	0.5
12 wk	1.4	<0.3	0.5	0.2	1	<1	9*	ND
24 wk	0.5	0.8	ND	0.2	1	0.1	0.3	ND
52 wk	1	2.4*	4*	ND	0.3	ND	0.4	0.3

ND indicates not done.
*Specimen may have been drawn less than 14 days after infusion.

Study population and vector administration

Each of the 8 subjects has a different missense mutation as the cause of his disease. This is typical for hemophilia B, where no single mutation predominates. Intramuscular injection of rAAV appears to be safe at the doses administered here. A consistent finding was the absence of any symptoms or signs of systemic illness during follow-up after vector injection. Most of the adverse events observed were related to trauma surrounding the intramuscular injections in subjects with bleeding disorders, or to the subsequent muscle biopsies performed to permit assessment of gene transfer and expression in this phase 1 study. Such a procedure would not be a routine part of therapeutic vector administration.

Biodistribution studies

A potential adverse event unique to gene transfer studies is the risk of inadvertent germline transmission of the donated DNA sequences. Because vector integration into germ cells, if it occurs, is likely to be random in the setting of rAAV, an integration event could potentially have disastrous effects for progeny conceived from such a germ cell. Thus, a working guideline is that germline transmission of vector DNA should be avoided.[40-42] Biodistribution studies performed here document that there is no evidence of vector DNA in semen samples obtained from subjects at any time point following vector injection. Thus, the risk of inadvertent germline transmission of vector sequences would appear to be very low for doses up to 1.8×10^{12} vg/kg delivered to skeletal muscle. See "Appendix" for additional notes on the biodistribution studies.

Absence of antibodies to F.IX

A major safety concern in any novel treatment for hemophilia is the risk of developing inhibitory antibodies to the clotting factor.[49,50] Subjects with a previous history of inhibitory antibodies formed in response to infused F.IX protein were excluded from this study. However, data generated in animal models suggest that antigen processing and presentation of F.IX may differ in protein infusion approaches versus gene transfer approaches.[51-53] In the studies shown here, there was no evidence of formation of either inhibitory or noninhibitory antibodies following vector injection. Two specific exclusion/inclusion criteria may have been key to this safety feature. First, we limited enrollment to individuals with missense mutations; those with nonsense mutations, gene inversions, or gene deletions were excluded from participation. The rationale for this was based on studies in 2 different hemophilia B dog models.[23,28,54] Animals with missense mutations generally did not form inhibitory antibodies to canine F.IX after intramuscular vector injection (except at high doses), whereas animals with an early stop codon routinely developed inhibitory antibodies to AAV vector–encoded canine F.IX even at low doses. This finding is consistent with older observations[55] derived from studies of patients with hemophilia B treated with clotting factor concentrates, which demonstrated that individuals with missense mutations virtually never develop inhibitory antibodies, whereas those with mutations that result in substantial loss of coding information (eg, gene deletions, early stop codons) have a risk of inhibitor formation considerably higher than the hemophilia population as a whole. It is important to note that, in dogs with hemophilia B due to an early stop codon, inhibitory antibodies could be elicited even at vector doses too low to result in detectable circulating levels of F.IX. Thus for individuals with mutations that result in a substantial loss of coding information, there is a risk of inhibitor formation even at low doses of vector.

The second key feature in avoiding inhibitor formation was a strict limitation on the dose of vector injected at each site. In earlier studies in hemophilic dogs, we had shown that the risk of generating inhibitory antibodies increased with increasing vector dose per site.[28] A change in the dose per site changes several variables that may affect antigen presentation; the total number of viral particles rises, the amount of antigen produced per site rises, and the level of any contaminant in the vector preparation also rises. Whatever the mechanisms involved, dog studies suggest that keeping the dose per site below 2×10^{12} vg reduces the risk of inhibitory antibody formation.

Antibodies to AAV

A potential obstacle to therapy with rAAV is the presence in a substantial portion of the human population of neutralizing antibodies to the wild-type AAV capsid.[56] Thus one goal of these studies was to determine whether these antibodies block gene transfer and expression with a rAAV vector. Comparison of the data in Table 7 and Table 8 suggests that transduction is not blocked, because subjects with high-titer pretreatment neutralizing antibodies (subjects A, B, D, E, and G) all had evidence on muscle biopsy for gene transfer or expression or both. See "Appendix" for additional notes on neutralizing antibodies to AAV.

Evidence for gene transfer and expression on muscle biopsy

Analysis of transduced muscle tissue has afforded the opportunity to determine how accurately studies in hemophilic dogs have predicted results in humans. The findings reported here in this first human study are remarkably similar to those we reported in studies in the large animal model.[23] In both cases, vector DNA is detectable on Southern blot of injected tissue as a high-molecular-weight form, gene copy number is about 0.5 to 4 copies/diploid genome at doses of about 1.5×10^{12} vg/site, and immunohistochemistry shows the same checkerboard pattern of positively staining fibers directly adjacent to negative fibers.

A puzzling feature of early studies of muscle-directed gene transfer with rAAV was the universally noted mosaic-like pattern of transgene expression.[17,57] Huard and colleagues provided evidence in studies in mice to suggest that this pattern reflects differences in the abundance of HSPG on slow and fast muscle fibers,[30] and we show here that the same explanation applies in human muscle. This finding had an important consequence for the clinical study, because the site initially selected for muscle injection, the vastus lateralis muscle, chosen for its ease of access for both injection and biopsy, is one with a moderately low slow fiber content. This discovery resulted in a modification to the clinical study, to include as injection sites the deltoids and the soleus, where slow fibers comprise 61% and 85% of the muscle, respectively.[32]

Need for higher doses and practical limitations to dose escalation

As reported previously and as shown in Table 10, vector doses of 1.8×10^{12} vg/kg reliably yield circulating F.IX levels of more than 1% in mice, whereas 4- to 5-fold higher doses (8.5×10^{12} vg/kg) are required for levels more than 1% in dogs. At the outset, it was unclear whether mice or dogs would more accurately predict dose response in humans, but based on data from this phase 1 study, it is clear that doses of 1.8×10^{12} vg/kg do not yield levels of more than 1% F.IX in humans and that higher doses will be required. A major limitation to dose escalation, however, is the need to inject larger numbers of sites as the dose is increased. This requirement rests on 3 distinct features of AAV-mediated gene transfer and F.IX expression in skeletal muscle. First, F.IX undergoes extensive

Table 10. AAV-F.IX muscle results in 3 species

Dose, vg/kg	Mice*	Dogs†	Peak human
2×10^{11}	<1%	<0.1%	1.4%, 0.8%, <1%
6.0×10^{11}	<1%	0.2%	<1%, 1%, <1%
1.8×10^{12}	1.5%	0.2%-0.4%	<1%, <1%
4.0×10^{12}	3%	0.4%	ND
8.5×10^{12}	ND	1.4%	ND
1.6×10^{13}	5%-7%	ND	ND

ND indicates not done.
*See Herzog et al.[22]
†See Herzog et al.[23]

posttranslational modification, and skeletal muscle has only a limited capacity to accurately and efficiently execute these changes. At high levels of synthesis, biologically inactive material is secreted.[8] Second, in studies in large animal models, we have shown that the risk of inhibitor formation in hemophilic dogs increases with increasing dose per site, so that avoidance of this complication requires injection of progressively higher numbers of sites as the dose is raised.[28,58] Finally, there is a theoretical limitation on dose per site for receptor-mediated uptake of vector. Note that the use of alternate serotypes that transduce muscle more efficiently[59,60] may circumvent the third limitation, but would have no effect on the other 2.

Absence of a clear dose-response effect

At the vector doses administered in this trial, efficacy was quite limited, with 2 of 8 subjects demonstrating a small elevation of F.IX levels ($\geq$ 1% but < 2%), and 2 of 8 subjects reducing the use of F.IX concentrate by at least half for periods of more than 1 year. Because dose escalation was stopped at a dose considerably lower than we had originally proposed (Table 1), it is perhaps not surprising that a clear dose-response relationship could not be demonstrated. Several groups have reported methods for measuring very low levels of circulating F.VIII on plasma samples; it will be of interest to determine whether these can also be extended to F.IX,[61,62] because more sensitive measures at low levels may permit analysis of whether a dose response is occurring.

Of interest is that a subject who received the lowest dose of vector achieved the highest level of expression (subject A). One factor that may have elevated this subject's circulating levels of F.IX was the coadministration of zidovudine. As we have previously shown,[63] the presence of zidovudine in the culture medium increases levels of transgene expression by as much as 30-fold in AAV-transduced cells for reasons that are not clear. Subject A is the only one who was taking zidovudine, which may account at least in part for his better than average response. Note that lot-to-lot variation in vector preparations should not be a factor in the absence of a clear dose response, because each lot undergoes potency testing through transduction of cultured cells and is not released for clinical use unless the amount of F.IX produced per vector genome falls within a specified range.

Summary

In this report we have demonstrated that intramuscular injection of an AAV vector encoding human F.IX was well tolerated with no significant systemic or local toxicities in 8 human subjects. Vector was not detected in the semen at any time point, consistent with animal data suggesting a low risk of vertical transmission of rAAV vector given by this route. Antibodies to F.IX were not detected, either by Bethesda assay or by Western blot. PCR analysis, Southern blot, and immunohistochemistry of biopsied injected muscle provided clear evidence of gene transfer and expression following intramuscular injection of AAV-F.IX. Although circulating levels of F.IX were less than what is required for a therapeutic effect, the study illustrates that the general characteristics of AAV transduction in skeletal muscle are similar in animals and humans. The data reported here are the foundation for ongoing studies in which AAV-F.IX is introduced into the liver in patients with severe hemophilia B. As secretion of F.IX from hepatocytes is much more efficient than secretion from muscle cells, it may be possible to achieve therapeutic levels with this approach in humans, as has already been established in hemophilic dogs.[48] In addition, these findings have important implications for treatment of muscular dystrophies[64] or for other diseases where lower levels of protein secretion from skeletal muscle are adequate for a therapeutic effect.[65,66]

Appendix

Notes on biodistribution studies

Generally, the biodistribution studies demonstrate that vectors can be detected in serum, saliva, and urine for 24 hours after intramuscular administration of the vector and only occasionally thereafter. In a recent study by Favre et al,[67] the authors demonstrated that white blood cell (WBC) DNA was positive for vector sequences up to 9 months following intramuscular injection of rAAV in nonhuman primates. Although WBC DNA was not specifically analyzed in this study, this is the likely explanation for the positive serum sample at 12 weeks in subject E. Another important point demonstrated in the Favre et al[67] study is that infectious AAV was never detected in body fluids obtained more than 3 days after injection, suggesting that the PCR-detectable sequences isolated at later time points do not represent a risk for horizontal transmission. See "Biodistribution studies" in "Discussion" for additional information.

Notes on anti-AAV antibodies

Infection with wild-type AAV occurs via the respiratory tract; whether the neutralizing antibodies measured in the in vitro assay described here have any predictive value for transduction of skeletal muscle (or liver) is not clear. In this study, more than 10^{12} vector genomes were delivered to each injection site. It is likely that such high local particle concentrations would overwhelm even a high-titer neutralizing antibody. See "Antibodies to AAV" in "Discussion" for additional information.

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Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy

Jane D. Mount, Roland W. Herzog, D. Michael Tillson, Susan A. Goodman, Nancy Robinson, Mark L. McCleland, Dwight Bellinger, Timothy C. Nichols, Valder R. Arruda, Clinton D. Lothrop Jr, and Katherine A. High

Hemophilia B is an X-linked coagulopathy caused by absence of functional coagulation factor IX (FIX). Using adeno-associated virus (AAV)–mediated, liver-directed gene therapy, we achieved long-term (> 17 months) substantial correction of canine hemophilia B in 3 of 4 animals, including 2 dogs with an FIX null mutation. This was accomplished with a comparatively low dose of 1 × 10^{12} vector genomes/kg. Canine FIX (cFIX) levels rose to 5% to 12% of normal, high enough to result in nearly complete phenotypic correction of the disease. Activated clotting times and whole blood clotting times were normalized, activated partial thromboplastin times were substantially reduced, and anti-cFIX was not detected. The fourth animal, also a null mutation dog, showed transient expression (4 weeks), but subsequently developed neutralizing anti-cFIX (inhibitor). Previous work in the canine null mutation model has invariably resulted in inhibitor formation following treatment by either gene or protein replacement therapies. This study demonstrates that hepatic AAV gene transfer can result in sustained therapeutic expression in a large animal model characterized by increased risk of a neutralizing anti-FIX response. (Blood. 2002;99: 2670-2676)

Introduction

Hemophilia B is a sex-linked bleeding disorder caused by a deficiency of functional coagulation factor IX (FIX). Current replacement therapy consists of intravenous infusion of protein concentrate. However, this treatment is costly and inconvenient and carries with it the risk of blood-borne disease transmission. Furthermore, bleeds are often treated only after they have occurred, rather than prophylactically, so that chronic joint damage occurs and the risk of a fatal bleed is always present. Hemophilia is an ideal model for gene therapy because precise regulation and tissue-specific transgene expression are not required.[1,2] A number of animal models are available including knockout mice and well-described hemophilic dog colonies with phenotypes corresponding to the human disease.[3-5] Clinical end points for treatment are well defined. An increase of factor levels to more than 1% will improve the phenotype of the disease from severe to moderate, with reduced frequency of spontaneous bleeds, and a further increase to more than 5% will result in a mild phenotype; that is, patients would likely require factor infusion only after severe injury or during surgery. Currently the most serious complication of treatment is the formation of inhibitory antibodies to the deficient protein, which occurs with a frequency of 3% to 4% in patients with hemophilia B.[6,7] Inhibitor formation is observed mostly in those patients with extensive loss of FIX coding information.[6,8]

Sustained expression of canine FIX (cFIX) in dogs with a missense mutation has been observed following administration of an adeno-associated virus (AAV) vector into the portal vein for hepatic gene transfer or into skeletal muscle.[9-11] The latter approach is currently being tested in a phase 1 clinical trial.[12] AAV vectors can be produced in a helper virus-free system, are devoid of any viral gene products, and often fail to activate antigen-specific cytotoxic T lymphocytes.[13] However, inhibitor formation is still a frequent complication following intramuscular administration of AAV vector in hemophilia B mice (with a large *F9* gene deletion) and dogs with a FIX null mutation.[14,15] In these animal models, muscle-directed gene therapy was successful only when combined with transient immunosuppression. Inhibitor formation in the null mutation dogs has also been described in the context of lentiviral transfer of a *cF9* gene to the liver.[16] These data underscore a potentially serious immunologic complication for all gene replacement strategies for treatment of genetic disease, that is, a harmful immune response to the transgene product in a recipient who is not tolerant to the therapeutic protein encoded by the donated gene.

In this study, we show sustained correction of canine hemophilia B following AAV-mediated, liver-directed gene transfer in the context of a FIX null mutation that is usually associated with a high risk of inhibitor formation in protein or gene therapy.[15,16] These data are encouraging for gene-based treatment and are directly relevant to a recently initiated clinical trial of AAV-mediated *F9* gene transfer to patients with severe hemophilia B.

From the Scott-Ritchey Research Center and Department of Clinical Sciences, College of Veterinary Sciences, Auburn University, AL; Departments of Pediatrics and Pathology, University of Pennsylvania Medical Center and The Children's Hospital of Philadelphia; and Department of Laboratory Medicine and Pathology, University of North Carolina, Chapel Hill.

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J.D.M. and R.W.H. contributed equally to the study.

Reprints: Katherine A. High, The Children's Hospital of Philadelphia, Abramson Research Center, Rm 310, 34th St and Civic Center Blvd, Philadelphia, PA 19104; e-mail: high@email.chop.edu.

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Materials and methods

AAV vector construction

Vector AAV-(ApoE)4/hAAT-cFIX was constructed by replacing the cytomegalovirus (CMV) enhancer/promoter in the previously described expression cassette with a liver-specific ApoE/hAAT enhancer/promoter combination.[9] This 1.1-kb sequence is comprised of the human α_1-antitrypsin promoter and 4 copies of the ApoE enhancer as described by Ponder and colleagues.[17] The expression cassette also contains a chimeric β-globin/CMV intron, the cFIX complementary DNA (cDNA), and the human growth hormone polyadenylation (hGH poly A) signal as described.[9] AAV2 vector was produced by triple transfection of HEK-293 cells in a helper virus-free system, which uses 2 helper plasmids to supply adenoviral gene functions (E2A, E4, and VA) and the AAV2 rep/cap genes.[18] Plasmids were grown in *Escherichia coli* DH5α cells and purified using the Qiagen (Santa Clarita, CA) Giga kit for preparation of endotoxin-free DNA. The AAV helper plasmid has been engineered to increase cap expression and to decrease generation of wild-type AAV to undetectable levels (< 1 in 10^9 vector particles) in a replication center assay.[19] AAV vector was purified from cell lysates by repeated rounds of CsCl density gradient centrifugation as described.[9,15,18] Vector was osmotically stabilized in Hepes-buffered saline, pH. 7.8, filter-sterilized, and stored at −80°C prior use. Vector titers were determined by quantitative slot blot hybridization. The Limulus amoebocyte lysate assay (Sigma, St Louis, MO) was performed to confirm absence of detectable endotoxin in vector preparations.

Experimental animals

The experimental animals used in this study were Lhasa Apso-Basenji cross dogs from the hemophilia B colony housed at the Scott-Ritchey Research Center at Auburn University. These dogs were males with severe hemophilia B caused by a 5–base pair (bp) deletion and a C>T transition in the *F9* gene that results in an early stop codon and unstable FIX transcript.[5] One of the dogs treated with the AAV vector also had pyruvate kinase (PK) deficiency, an erythrocyte metabolism disorder resulting in a low hematocrit of 20% as compared to normal levels of about 40%.[20] Additionally, a female hemophilia B dog with an FIX missense mutation of the University of North Carolina (UNC)–Chapel Hill colony was treated.[3] All animals were housed in US Department of Agriculture–approved facilities and the experimental protocol was approved by the institutional Animal Care and Concern Committees of Auburn University and UNC-Chapel Hill.

Vector administration

The animals were premedicated with diazepam (5 mg) or butorphanol (5 mg) or both and atropine (0.6 mg) before anesthetic induction with isoflurane. A midline laparotomy was performed: a mesenteric vein was then isolated and a 20-gauge catheter inserted and tied off with stay sutures. The AAV-$(ApoE)_4$/hAAT-cFIX vector (in a 10-mL volume) was administered by slow bolus infusion (1-2 minutes) and the catheter flushed with 5 to 10 mL heparinized saline before removal and ligation of the mesenteric vein (mesenteric vein administration results in subsequent delivery of the vector to the portal vein for hepatic gene transfer). The abdomen was closed using standard surgical procedures. Butorphanol was administered as needed to provide postoperative analgesia. The dogs were prophylactically administered 90 mL plasma immediately before surgery and 45 mL 8 to 12 hours later. Abnormal reactions or toxicity were not noted following vector administration based on clinical examination and routine clinical pathology tests. Portal vein infusion of vector in hemophilia B dog E34 following midline laparotomy was preformed as described previously.[10,11] Vector administration by this method was well tolerated in this animal as well.

FIX, coagulation, and antibody assays

Blood samples were drawn from hemophilia B dogs as described.[15] The whole blood clotting time (WBCT), activated clotting time (ACT), activated partial thromboplastin time (aPTT) of plasma samples, and FIX activity levels were measured as previously reported.[9,15] The cFIX antigen levels in plasma samples were determined by enzyme-linked immunosorbent assay (ELISA).[9,15] Anti-cFIX was demonstrated by immunocapture assay specific to canine IgG1, IgG2, IgM, and IgA, by Western blot, or by Bethesda assay as described previously.[14,15] One Bethesda unit (BU) represents inhibition of normal FIX activity by 50%. The cFIX protein used in these assays was a purified plasma-derived preparation from Enzyme Research Laboratories (South Bend, IN), and all antibodies were purchased from Bethyl Laboratories (Montgomery, TX).[15] Antiphospholipid was detected by dilute Russell viper venom time (RVVT). Neutralizing antibodies (NABs) against AAV2 vector particles were measured by inhibition of in vitro LacZ transduction as described.[15] The treated animals did not have a pre-existing NAB titer, but all developed NABs to AAV2 after vector administration.

DNA analysis

Total genomic DNA was isolated from canine liver or spleen tissue using the Easy DNA kit from Invitrogen (Carlsbad, CA). Vector-specific sequences were detected by Southern blot hybridization using a 0.9-kb probe specific to the human α_1-antitrypsin promoter and intron sequences in the AAV vector.

Results

Administration of liver-specific AAV vector results in sustained high-level FIX expression in 3 hemophilia B dogs and transient expression in 1 dog

AAV-$(ApoE)_4$/hAAT-cFIX vector was infused into the hepatic circulation of 4 hemophilia B dogs (via mesenteric or portal vein) for hepatocyte-specific expression of cFIX. The vector uses a strong liver-specific promoter/enhancer combination.[17] Two animals from the Auburn dog colony (Brad and Semillon) and 1 animal from the UNC-Chapel Hill colony (E34) received vector at a dose of $\sim 1 \times 10^{12}$ vector genomes (vg)/kg (Table 1). Before treatment none of these animals had detectable circulating cFIX antigen or cFIX activity owing to a FIX null mutation (5-bp deletion plus a C>T transition in the *F9* gene resulting in an early stop codon at amino acid residue 146 before the activation peptide of FIX, Auburn dogs) or a FIX missense mutation (G>A resulting in a single amino acid substitution of glutamic acid for Gly379 in the catalytic domain of FIX, UNC dog).[3,5]

Brad, the first dog treated, also received a total of 180 mL plasma on day 0 before, during, and after surgical laparotomy and vector administration and 45 mL daily for the next 4 days. (All other animals received only ~135 mL plasma before and just after surgery.) By day 14, 10 days after the last plasma infusion, the ACT in Brad was 1.5 minutes (normal range, 1-2 minutes) as compared to 5.5 minutes the day before vector administration. The ACT has remained in the normal range for more than 17 months following vector administration (Figure 1B). During the same period, the WBCT was within the normal range (12.1 ± 2.6 minutes versus > 60 minutes before treatment), and aPTT values (29.4 ± 3.6 seconds) were significantly shortened from pretreatment times of 79.9 seconds (Figure 1A,C). The cFIX antigen was undetectable before vector administration but had increased to 317 ng/mL by week 2 and peaked at 907 ng/mL on week 16 (Figure 1D). Antigen levels of 590 ± 150 ng/mL have persisted for the duration of the study. Likewise, cFIX activity of 8.6% ± 2.1% of a canine plasma pool has also persisted for the more than 17-month observation period (Figure 1E and Table 1). The dog also had a normal cuticle bleed time after treatment (data nor shown). The other 2 dogs (E34 and Semillon) also showed sustained complete or nearly complete correction of the WBCT and ACT (not measured in E34) and

Table 1. Summary of dogs with hemophilia B treated by intramesenteric vein or portal vein (E34) administration of AAV-(ApoE)$_4$/hAAT-cFIX

Animal (colony)	Age* (mo)	Weight* (kg)	Total dose (vg)	Dose/kg (vg/kg)	PK deficiency	WBCT (min)	APTT (s)	cFIX (ng/mL)	cFIX activity (%)
Brad† (Auburn)	9	10.2	1.25×10^{13}	1.2×10^{12}	No	12 ± 2.5	29.5 ± 3.5	590 ± 150	8.5 ± 2
Semillon† (Auburn)	5.5	6.0	9.7×10^{12}	1.6×10^{12}	No	13.5 ± 4	35.5 ± 2	220 ± 65	5 ± 2.5
Beech† (Auburn)	12	10.5	3.6×10^{13}	3.4×10^{12}	Yes	≥ 10	≥ 36.2	≤ 2560	≤ 3
E34‡ (UNC)	5	12.3	9.6×10^{12}	8×10^{11}	No	11 ± 2.5	32 ± 4.5	262 ± 92	5 ± 2.5

Results for WBCT, aPTT, cFIX plasma levels as determined by ELISA, and cFIX activity as percentage of activity in normal dog plasma are average values (± 1 SD) for weeks 3 to 86 (Brad), weeks 3 to 42 (Semillon), or weeks 3 to 48 (E34) after vector administration. Values for Beech represent peak levels before inhibitor development. The ranges for coagulation times in normal dogs are 6 to 10 minutes (WBCT), 1 to 2 minutes (ACT), and 18 to 20 seconds (aPTT). In hemophilia B dogs they are more than 60 minutes (WBCT), more than 4 minutes (ACT), and more than 60 seconds (aPTT).

*Age and weight at time of vector administration.

†Male hemophilia B dogs of Auburn colony with FIX null mutation.

‡Female hemophilia B dog of UNC-Chapel Hill colony with FIX missense mutation.

substantial correction of the aPTT from more than 60 seconds before treatment to about 32 to 35 seconds (Table 1 and Figure 1A-C). The cFIX antigen levels averaged 220 ± 65 ng/mL for Semillon and 262 ± 92 ng/mL for E34 (Figure 1D). FIX activity averaged 4.9% ± 2.6% of normal canine plasma for Semillon and 5% ± 2.5% for E34 (Figure 1E and Table 1). Expression was sustained in both animals for more than 6 months in Semillon and more than 7 months in E34 (experiment ongoing).

None of the 3 successfully treated hemophilia B dogs described above had evidence for bleeds following vector administration (a total of 4.3 years of observation for all 3 animals, the UNC dog E34 has been followed for more than 1 year since gene transfer) or liver biopsy (see below). Although bleeding episodes are rarely observed in the Auburn dogs,[15] dogs of the UNC-Chapel Hill colony animals receive plasma infusion in response to a bleed on average 6 to 7 times per year, albeit with considerable variation among individual animals.[9]

A third null mutation dog, Beech, was injected with 3.4×10^{12} vg/kg (~3 times higher vector dose, Table 1). WBCT and ACT values were within the normal range after gene transfer (weeks 2-4), but returned to baseline by week 5 (Figure 1A,B). The aPTT results were consistent with these observations, showing decreasing values through week 4 (without ever achieving a normal value), but returning to a greater than pretreatment value of 90.4 seconds by week 5 (Figure 1C). The cFIX antigen level rose to more than 2 μg/mL by week 4 but had dropped to 13 ng/mL by week 5, and was undetectable by week 6 (Figure 1F). FIX activity showed a similar pattern, rising from 0% to 1.3% by week 2, peaking at 3.0% on week 3, and returning to 0% by week 5 (Figure 1E). As shown below, loss of systemic cFIX expression was due to formation of an inhibitory anti-cFIX that first emerged at week 5. The discrepancy between cFIX antigen levels measured by ELISA and cFIX activity levels in Beech are likely due to the presence of an antiphospholipid antibody in this animal (*vide infra*) as determined by RVVT assay as described before for a different animal of this colony.[15] At 11 weeks after vector administration, Beech developed a fatal intra-abdominal bleed, which, due to a lack of canine bypass reagents such as factor VIIa, could not be treated.

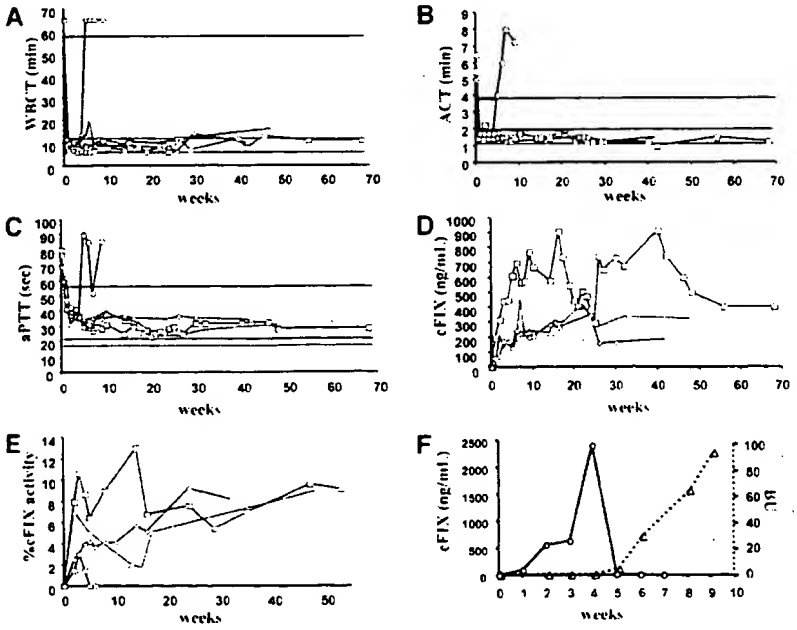

Figure 1. Coagulation parameters after vector administration. WBCT (A), ACT (B), aPTT (C), cFIX antigen levels in plasma (D), and cFIX activity levels (E; percent activity of pooled normal canine plasma) as a function of time after administration of AAV-ApoE-hAAT vector in hemophilia B dogs Brad (1×10^{12} vg/kg, □, blue line), Semillon (1×10^{12} vg/kg, ◇, green line), E34 (△, red line, ACT was not measured in this dog), and Beech (3×10^{12} vg/kg, ○, black line, data for Beech are omitted in graph D). Vector was administered into the mesenteric (Brad, Semillon, Beech) or portal vein (E34) for liver-directed gene transfer. The ranges for coagulation times in normal, healthy dogs are 6 to 10 minutes (WBCT), 1 to 2 minutes (ACT), and 18 to 20 seconds (aPTT). In hemophilia B dogs they are more than 60 minutes (WBCT), more than 4 minutes (ACT), and more than 60 seconds (aPTT). (F) The cFIX antigen levels (○) and formation of inhibitory anti-cFIX (in BU, △) in Beech after vector administration.

Antibody responses against cFIX

Beech, the dog with transient FIX expression, developed FIX-specific antibodies concomitant with the loss of FIX antigen and activity. The Bethesda titer increased from 0 (before treatment through week 4) to 4.0 BU at week 5 with a subsequently rising titer (Figure 1F). Anti-cFIX IgG was undetectable in serum from week 0 through 4, but was demonstrated in week 5 and subsequently by Western blot (Figure 2D). Immunocapture assay showed synthesis of IgM at week 4 followed by high titer IgG2 anti-cFIX at week 5 and low titer IgG1 at week 9 (Figure 2E). Brad, Semillon, and E34, the dogs with sustained FIX expression, had no evidence for anti-cFIX by Western blot, immunocapture assay, or Bethesda assay at any time point tested (Figure 2A-C and data not shown). IgA anti-cFIX was not detected in any of the treated animals (data not shown), and no animals had anti-cFIX before treatment.

Lack of vector-related toxicity

Serum chemistry panels showed no changes in liver (alanine aminotransferase [ALT]) enzyme tests for dogs Semillon, Beech, and E34 following vector administration ($1\text{-}3 \times 10^{12}$ vg/kg, data not shown). None of these animals experienced surgical complications and apparently tolerated vector infusion well. Brad had elevated ALT levels (4 times upper level of normal) during the first 3 to 5 days after vector administration as a result of a surgical complication. The dog had a hematoma at its right hind leg that was present before surgery. The resulting blood loss caused cardiac arrest during the procedure (before vector infusion), which required cardiopulmonary resuscitation including injection of atropine, epinephrine, and sodium bicarbonate to restore cardiac activity and normal circulation. Subsequently, vector was successfully infused without incident. Liver biopsies performed in Brad and Semillon 27 and 32 weeks after vector administration showed normal hepatic tissue without evidence of inflammation or other pathologic changes (Figure 3A-B). Brad and Semillon received a small amount of plasma (~50 mL) prior to liver biopsy and had no subsequent bleeds or other indication for requirement of additional FIX infusion. Hepatic tissue of Beech (necropsy performed 11 weeks after vector administration), the animal with PK deficiency, showed evidence for fibrosis (Figure 3C,F), early cirrhosis, and iron overload (Figure 3I, blue stain) due to chronic hemolytic anemia.

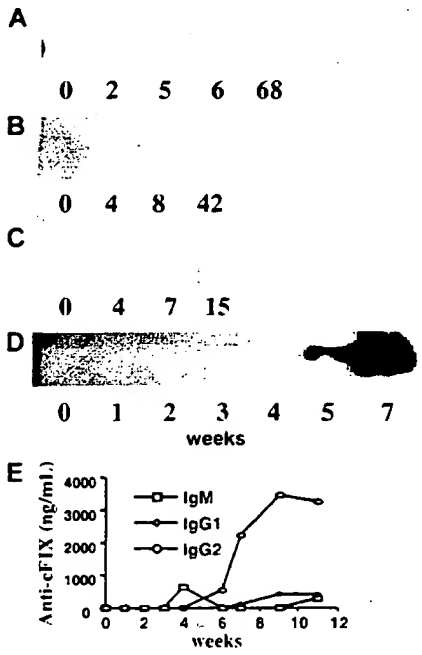

Figure 2. Western blot analysis after infusion of AAV vector. Western blot analysis demonstrating presence or absence of anti-cFIX IgG in hemophilia B dogs Brad (A), Semillon (B), E34 (C), and Beech (D) as a function of time after AAV vector was infused. Numbers indicate weeks after vector administration. (E) Serum levels of anti-cFIX immunoglobulins in Beech as a function of time after vector administration. Note that no anti-cFIX was detected in Brad, Semillon, or E34.

DNA analysis of vector sequences

Total genomic DNA from liver and spleen tissue obtained from Beech at necropsy was analyzed by Southern blot hybridization. Using a restriction enzyme (*Eco*RI) that releases a defined, vector-specific fragment of 0.9 kb, we estimated a gene copy number of 0.1 copies per diploid genome in the liver after comparison of signal strength with plasmid standards (Figure 4B, lanes 1-4). No signal was obtained from splenic DNA (Figure 4B, lane 7). A small amount of episomal double-stranded vector (monomer) was observed in uncut liver DNA (lane 6). Most of the vector was present as a high-molecular-weight species, at least in part arranged as head-to-tail concatemers (digest with enzyme *Nco*I that cuts once within the vector, thereby releasing a fragment of the size of the unit length of the vector, lane 5). These results are consistent with previous observations for AAV-mediated gene transfer to liver and skeletal muscle.[21,22]

Discussion

The FIX-deficient dogs in the Auburn colony have been previously shown to rapidly produce high-titer inhibitors to cFIX (within 9-14 days) following IV infusion of purified cFIX or transduction of skeletal muscle with a cFIX-encoded AAV vector (1×10^{12} vg/kg) indicating that the animals are at high risk for inhibitor formation.[15] Similarly, inhibitor formation has been reported in other liver-directed gene transfer strategies in these dogs.[16] This strain of hemophilia B dogs may therefore be an excellent large animal model to mimic the 3% to 4% of hemophilia B patients who develop inhibitors in conventional protein replacement therapy. As is the case in many FIX-deficient patients with inhibitors, these animals are not tolerant to FIX because of the severity of their mutation, which causes lack of synthesis of cFIX antigen in the liver.[5] Consequently, a neutralizing anti-cFIX response blocked systemic expression in dogs of this colony after muscle-directed gene therapy at vector doses that conferred sustained expression of cFIX in hemophilia B dogs with a FIX missense mutation (inhibitor formation in 2 of 2 null mutation dogs and 0 of 4 missense mutation dogs at dose $\leq 3 \times 10^{12}$ vg/kg).[9,15] To our surprise, we were able to achieve sustained expression of cFIX even in hemophilia B dogs of the Auburn colony (at levels that result in substantial correction of the bleeding disorder) using hepatic gene transfer.

Efficacy of hepatic gene transfer and expression

In treatment of hemophilia, quantitative differences in factor levels translate into qualitative differences in improvement of the disease phenotype. Previous studies in the missense mutation model of canine hemophilia B have demonstrated sustained expression of up to about 1% of normal levels in muscle- or liver-directed gene therapy with AAV vectors, whereas other gene transfer strategies

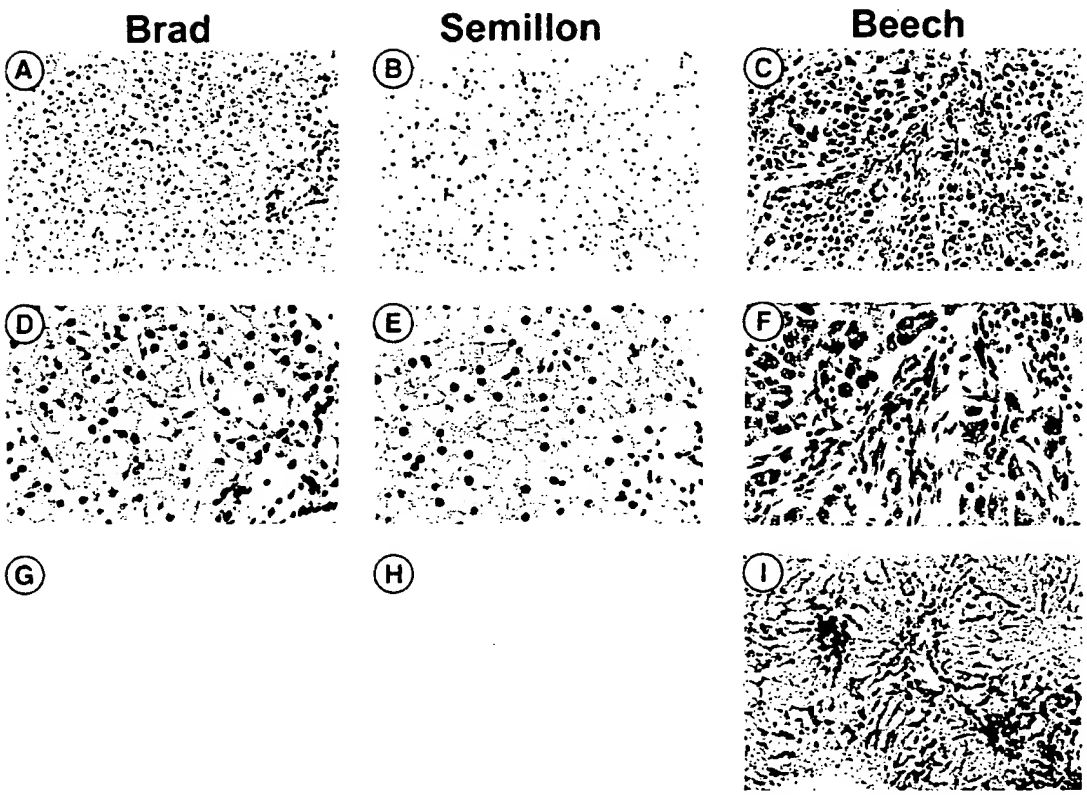

Figure 3. Histologic examinations. Histology of formalin-fixed liver sections from hemophilia B dogs (with FIX null mutation) Brad (A, D, G), Semillon (B, E, H), and Beech (C, F, I) treated with the AAV-$(ApoE)_4$/hAAT-cFIX vector. The tissues were stained with hematoxylin and eosin (H&E, A-F) or Prussian blue (PB), a stain for detection of iron (G-I). Tissues from Brad and Semillon were taken by biopsy at week 27 and 32, respectively, and tissue from Beech was taken by necropsy at week 11. Beech had PK deficiency, a disease characterized by iron deposition in the liver as evident by the blue stain in panel I. Fibrosis (note fibrotic changes in panels C and F) is present in panels C and F but not in panels A, B, D, and E. Original magnification × 100 (A-C and G-I) or × 400 (D-F).

had yielded only subtherapeutic or transient cFIX expression.[23,24] In another recent study on liver-directed gene transfer with AAV, vector doses of 5×10^{12} vg/kg resulted in expression of 0.5% to 4% of normal levels in the missense mutation dogs.[11] Compared to the latter, expression levels per delivered vector particle were 5- to 15-fold higher in the studies presented here and 10- to 50-fold higher than in our previous studies on muscle-directed gene transfer in canine hemophilia B. These results are encouraging for clinical application in humans, because high levels of expression are achievable with relatively low vector doses, and the scale-up from the dog model to humans is minimal as opposed to data from mouse studies. Expression levels reported here should be adequate for substantial or nearly complete correction of the bleeding disorder in patients.

Lack of inhibitor formation after hepatic gene transfer

Use of species-specific transgenes allows us to define the risk of a neutralizing antibody response against expressed FIX antigen. For muscle-directed gene therapy, we have identified the choice of vector, vector dose, and the underlying FIX mutation as important factors that influence the risk of immune responses (Herzog et al[9]; Fields et al[14]; Herzog et al[15]; Fields et al[25]; and R.W.H., P. A. Fields, T.C.N., K.A.H., unpublished data, May 2000). In this study, we show that an alternative route of administration and choice for target tissue of transgene expression, namely liver, can allow systemic expression of FIX antigen in the context of an unfavorable mutation. Sustained expression of human FIX has been documented in hemophilia B mice with a large *F9* gene deletion resulting in absence of endogenous FIX.[10] However, hemophilic mice were bred on a C57BL/6 background and have also been successfully treated by systemic administration of a highly immunogenic first-generation adenoviral vector, a result that is not reproducible in other strains of mice.[26,27] Nonetheless, route of administration is an important determinant of the risk of a humoral immune response to a secreted transgene product, although the immunologic mechanisms are not understood at this point.[26,28,29]

The results from the Auburn dogs raise the question of whether liver-directed gene transfer may have the potential to induce tolerance to the expressed FIX antigen even in the absence of immune modulation. Induction of immune tolerance by gene transfer will be crucial for treatment of hemophilic patients who had not been extensively treated with coagulation factor antigen, if gene therapy were ever to be used for treatment of children with hemophilia. Based on our results on muscle-directed gene transfer (rapid induction of a T-helper cell-dependent antibody response[15,25]), one would expect activation of T-cell responses against the transgene product to follow a substantially different mechanism in the context of hepatic gene transfer. This likely reflects differences in the population of antigen-presenting cells in these tissues. For example, liver sinusoidal endothelial cells have been shown to confer antigen-specific $CD8^+$ T-cell tolerance and have also been implemented

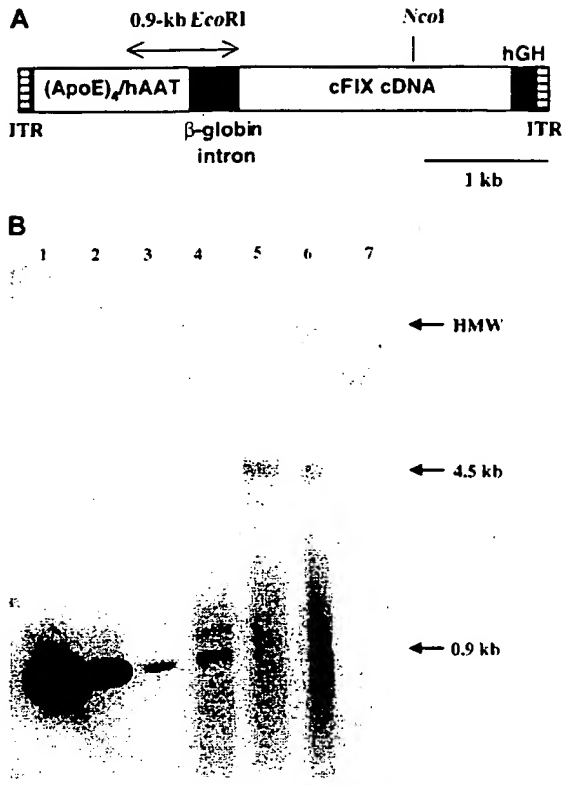

Figure 4. DNA analysis of vector sequences. (A) Diagram of AAV-(ApoE)$_4$/hAAT-cFIX vector. Shown are AAV2 inverted terminal repeats (ITRs), a 1.1-kb enhancer/promoter sequence containing 4 repeats of the human ApoE enhancer linked to the human α_1-antitrypsin promoter, a chimeric β-globin/CMV intron, the cFIX cDNA, and hGH poly A signal. A 0.9-kb *Eco*RI fragment used as a probe for Southern hybridization and a unique *Nco*I restriction site are also indicated. (B) Southern blot hybridization. Lanes 1-3: plasmid pAAV-(ApoE)$_4$/hAAT-cFIX encoding the vector, *Eco*RI digested, 1 ng, 100 pg, and 10 pg, respectively. Lanes 4-6: genomic DNA isolated from liver of hemophilia B dog Beech, 40 μg/lane. Lane 4, *Eco*RI digest; lane 5, *Nco*I digest; lane 6, uncut DNA. Lane 7: genomic DNA isolated from spleen, 40 μg, cut with *Eco*RI. Indicated on the right margin are high-molecular-weight DNA (HMW), the 4.5-kb fragment representing the unit length of the double-stranded vector, and the 0.9-kb *Eco*RI fragment used for estimation of gene copy number.

in CD4$^+$ T-cell tolerance, although the latter is less clear.[30,31] Antigen presentation in oral tolerance has been documented to result in immune deviation causing synthesis of IgA instead of IgG and consequently inefficient clearance of the antigen.[32] However, we found no evidence for IgA anti-cFIX in serum samples from our treated dogs. Interestingly, both Brad and Semillon have been subjected to liver biopsy, and neither dog has shown an anti-cFIX response indicating that immunologic unresponsiveness was maintained even after an invasive procedure. The liver has been shown to confer antigen-specific tolerance in several experimental models including portal vein tolerance, oral tolerance, and liver allotransplants across incompatible major histocompatibility complex barriers (which cannot easily be achieved for other organs), and may emerge as a preferred target for maintaining tolerance to a systemic transgene product as well.[32-35] The AAV vector with reduced potential for inflammation and increased capacity for sustained expression may be ideal for this purpose. It is likely that every combination of target tissue and vector is characterized by a distinct set of immunologic signals.

In one dog (Beech), inhibitor formation occurred, although much delayed compared with our results from muscle-directed gene transfer. Beech, unlike the other 2 dogs, also had PK deficiency, which causes chronic hemolytic anemia and an iron overload syndrome. Following necropsy, some level of fibrosis and early stages of cirrhosis were observed in liver tissue as expected in a PK-deficient animal. There was no obvious toxicity/liver pathology that may be attributed to vector treatment. It is unclear whether the PK deficiency, which may be associated with antibodies against red blood cells or antiphospholipid in this particular breed of dogs, poses an additional risk factor for inhibitor formation.[15,20] Alternatively, liver pathology secondary to iron overload (and thus altered local cytokine milieu), perhaps in combination with increase in vector dose, may have contributed to inhibitor formation in an animal already inhibitor prone because of the null mutation. Interestingly, elevated liver enzyme levels and other toxicity caused by surgical complications in hemophilia B dog Brad (likely including significant changes in cytokine expression that may provide strong activation or "danger" signals to the immune system) did not predispose to inhibitor formation. This suggests that transient liver toxicity caused by cardiopulmonary arrest, and chronic liver pathology caused by PK deficiency, are not comparable events from an immunologic viewpoint.

In conclusion, the data presented here are encouraging for treatment of patients with hemophilia B by in vivo hepatic gene transfer with an AAV vector. Treatment was successful in 3 of 4 large animals with severe disease including 2 of 3 animals of a strain that is prone to inhibitor formation due to a FIX null mutation. Moreover, expression levels were not only therapeutic, but near the curative range at low vector doses.

Acknowledgments

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EXHIBIT C

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NONVIRAL TRANSFER OF THE GENE ENCODING COAGULATION FACTOR VIII IN PATIENTS WITH SEVERE HEMOPHILIA A

DAVID A. ROTH, M.D., NICHOLAS E. TAWA, JR., M.D., PH.D., JOANNE M. O'BRIEN, R.N., DOUGLAS A. TRECO, PH.D., AND RICHARD F SELDEN, M.D., PH.D., FOR THE FACTOR VIII TRANSKARYOTIC THERAPY STUDY GROUP

ABSTRACT

Background We tested the safety of a nonviral somatic-cell gene-therapy system in patients with severe hemophilia A.

Methods An open-label, phase 1 trial was conducted in six patients with severe hemophilia A. Dermal fibroblasts obtained from each patient by skin biopsy were grown in culture and transfected with a plasmid containing sequences of the gene that encodes factor VIII. Cells that produced factor VIII were selected, cloned, and propagated in vitro. The cloned cells were then harvested and administered to the patients by laparoscopic injection into the omentum. The patients were followed for 12 months after the implantation of the genetically altered cells. An interim analysis was performed.

Results There were no serious adverse events related to the use of factor VIII–producing fibroblasts or the implantation procedure. No long-term complications developed, and no inhibitors of factor VIII were detected. In four of the six patients, plasma levels of factor VIII activity rose above the levels observed before the procedure. The increase in factor VIII activity coincided with a decrease in bleeding, a reduction in the use of exogenous factor VIII, or both. In the patient with the highest level of factor VIII activity, the clinical changes lasted approximately 10 months.

Conclusions Implantation of genetically altered fibroblasts that produce factor VIII is safe and well tolerated. This form of gene therapy is feasible in patients with severe hemophilia A. (N Engl J Med 2001; 344:1735-42.)

HEMOPHILIA A, an X-linked hemorrhagic disorder due to mutations in the gene that encodes factor VIII, affects 1 in 5000 males.[1] Approximately 60 percent of patients with a mutant gene for factor VIII have severe hemophilia (in which the level of factor VIII activity is less than 1.0 percent of normal), whereas the remainder have moderate or mild hemophilia (factor VIII activity, 1.0 to 5.0 percent of normal or more than 5.0 percent of normal, respectively).[1] In severe hemophilia, spontaneous bleeding into joints, soft tissues, and vital organs is frequent, whereas in mild hemophilia, bleeding usually occurs only after trauma or surgery.[2]

Replacement with intravenously administered concentrates of factor VIII controls bleeding in patients with hemophilia A. In patients with severe disease, spontaneous hemorrhage and progression of arthropathy can be prevented by prophylactic administration of exogenous factor VIII to maintain factor VIII activity levels above 1.0 percent of normal.[3] Prophylaxis with factor VIII is costly,[1] however, and does not guarantee the prevention of hemorrhage.[4] Despite considerable improvements in the safety of plasma-derived and recombinant factor VIII concentrates, concern remains about contamination with infectious prions[5,6] and small nonenveloped viruses, such as hepatitis A virus[7,8] and parvovirus.[9-11] Hemophilia A is a suitable candidate disease for gene therapy[12] for several reasons: factor VIII production is not regulated in response to bleeding; the broad therapeutic index of factor VIII minimizes the risk of overdoses; delivery of factor VIII into the bloodstream does not require expression of the gene by a specific organ; and even low levels of the protein can be beneficial.

From the Center for Hemostasis and Thrombosis Research, Department of Medicine (D.A.R., J.M.O.), and the Department of Surgery (N.E.T.), Beth Israel Deaconess Medical Center and Harvard Medical School, Boston; and Transkaryotic Therapies, Cambridge, Mass. (D.A.T., R.F.S.). Address reprint requests to Dr. Roth at Beth Israel Deaconess Medical Center, Center for Hemostasis and Thrombosis Research, RE-302, 41 Ave. Louis Pasteur, Boston, MA 02115, or at droth@caregroup.harvard.edu.

We have developed a nonviral gene-delivery system termed "transkaryotic implantation." It entails the isolation of somatic cells from a patient, the stable introduction of a therapeutic gene into these cells, the isolation and clonal propagation of a single engineered cell, and the implantation of the clonal cells into the patient.[13-15] Mice that received fibroblast implants carrying DNA sequences of the human factor VIII gene produced the human protein; the procedure was safe and resulted in factor VIII activity levels that exceeded 5.0 percent of normal — a level that would be considered therapeutic in patients with severe hemophilia A — for more than one year (unpublished data). These results led us to study the safety of the transkaryotic-implantation system in patients with severe hemophilia A.

METHODS

Patients

The study was designed as a single-institution, open-label, phase 1 trial in which autologous fibroblasts that produced human factor VIII were administered to patients with severe hemophilia A. Patients were eligible to be included in the study if they had hemophilia A with a level of factor VIII activity below 2.0 percent of normal; were at least 15 years old; had received at least 50 days of factor VIII therapy before study entry; had six or more bleeding episodes per year; had normal factor VIII clearance; had a hemoglobin level above 12 g per deciliter (7.4 mmol per liter); and had a platelet count above 100,000 per cubic millimeter. Patients with any of the following characteristics were excluded: the presence of a factor VIII inhibitor, as determined by the Bethesda inhibitor assay (which detects antibodies that neutralize factor VIII activity) at the time of enrollment; a history of inducible factor VIII inhibitor; an anticipated requirement for fixed-dose prophylaxis with factor VIII infusions during the study; a history of opportunistic infection or cancer related to the acquired immunodeficiency syndrome; use of investigational therapy for hemophilia within 30 days before enrollment in the study; or clinical evidence of abdominal adhesions or portal hypertension (which would increase the risk associated with the laparoscopic procedure).

The clinical protocol and the informed-consent document were approved by the Center for Biologics Evaluation and Research of the Food and Drug Administration and by the Office of Biotechnology Activities of the National Institutes of Health. Local review and approval were obtained from the Beth Israel Deaconess Medical Center institutional review board, the Harvard University Biosafety Committee, and the Harvard University Human Gene Therapy Advisory Committee. Permission to treat up to 12 patients and to monitor them for up to two years was given. All the patients provided written informed consent.

Figure 1 shows the steps involved in the study. First, the patients underwent a clinical evaluation in which a complete medical history was obtained and a physical examination, blood and urine tests, electrocardiography, and chest radiography were performed. Patients received diary forms for use at home to record and describe all bleeding episodes and all infusions of factor VIII during the study. The date, time, and site of bleeding were recorded for each episode, as were the number of units of factor VIII administered with each infusion. The reason for factor VIII infusion was assigned to one of the following categories: spontaneous bleeding, injury-related bleeding, prophylaxis, and other reasons (including use for the skin biopsy, the laparoscopy, and other procedures).

Skin Biopsy

A full-thickness, elliptical skin-biopsy specimen (1.6 by 0.4 cm) was obtained from the medial surface of the upper arm after the patient received local anesthesia. Dermal fibroblasts were isolated for cell culture and subsequent plasmid transfection. All the patients received an infusion of factor VIII before the biopsy to increase their plasma factor VIII activity level to 100 percent of normal. Factor VIII was administered for an additional three or four days to maintain normal hemostasis.

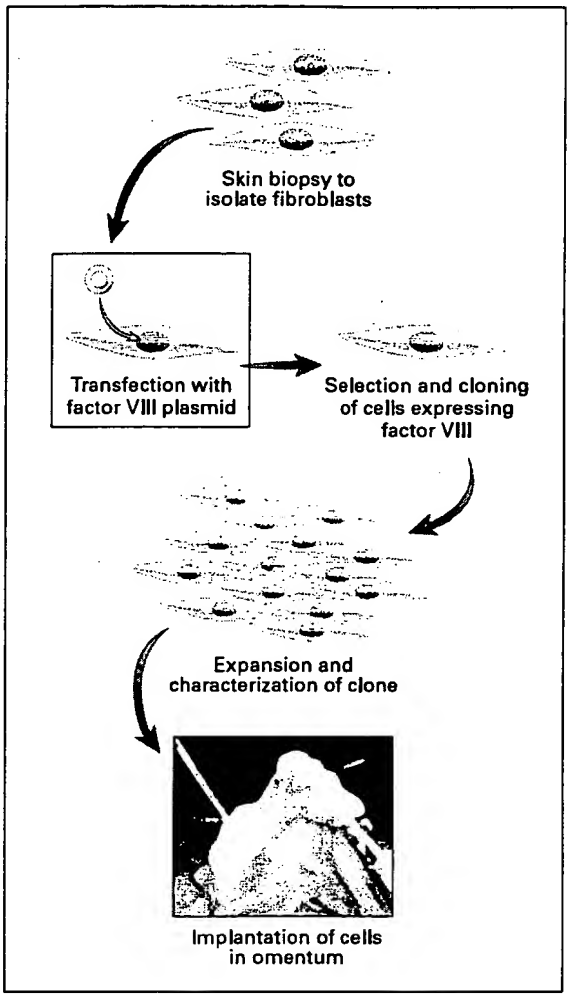

Figure 1. Steps in the Human Factor VIII Gene-Transfer Procedure. The fibroblasts from the skin-biopsy specimen were transfected with a plasmid containing the gene encoding human factor VIII from which the B domain had been deleted.

Laparoscopy

Approximately seven weeks after the skin biopsy, the patients returned to the hospital for another clinical assessment, which was performed the day before cell implantation. Factor VIII was infused just before surgery to increase plasma factor VIII activity levels to approximately 100 percent of normal, and the infusions were continued two or three times daily for one week after surgery. The laparoscopic procedure was performed while the patients were under general anesthesia. A 10-mm trocar was inserted into the peritoneal

cavity through an infraumbilical incision with the use of an open technique. After carbon dioxide insufflation, a laparoscope was introduced at this site. Under direct vision, a single, 5-mm operating trocar was inserted into the midabdomen, and through this trocar, a laparoscopic grasper was used to manipulate the greater omentum. A 9-cm (3.5-in.), 20-gauge spinal needle was inserted directly through the abdominal wall under direct vision, and approximately 0.5 ml of the cell suspension was injected through the needle into the greater omentum at each of several sites. After surgery, the patients were observed overnight in the clinical research center. All six patients were discharged home on the first postoperative day.

Clinical assessments at the patients' homes were performed daily by the study nurse on days 2 through 6 after surgery. The patients were scheduled to undergo clinical assessments at the hospital during weeks 1, 2, 3, 4, 6, 8, 12, and 18 and months 6, 9, and 12 after cell implantation, with additional monitoring through year 2.

Preparation of Autologous Fibroblasts Expressing Factor VIII

A plasmid that contained the gene encoding human factor VIII from which the B domain had been deleted and that contained the human fibronectin promoter was introduced by electroporation into dermal fibroblasts that had been isolated from the skin-biopsy specimen. The B-domain coding sequence was not incorporated into the plasmid, because it is not required for the coagulant activity of factor VIII or the interaction of factor VIII with von Willebrand factor[16,17]; moreover, the presence of the B domain reduces expression of factor VIII in transfected mammalian cells. Fibronectin, an extracellular-matrix protein, is expressed in fibroblasts; its promoter efficiently directs the transcription of B-domain–deleted factor VIII in primary human dermal fibroblasts.

Stably transfected fibroblast clones that had incorporated the plasmid containing B-domain–deleted factor VIII were selected in G418-containing medium, isolated, expanded in nonselective medium, and characterized. Characterization included measurement of factor VIII expression, assessment of cell-growth properties (including soft-agar tumorigenicity assay in vitro), microbial safety, and Southern blotting. Cells from the clone that was designated for implantation were harvested the day before implantation, extensively washed, and introduced into a syringe. Either 100 million or 400 million cloned cells were then administered. During preparation, the fibroblast cultures were aseptically processed in class 100 conditions.[18]

Factor VIII Assays

Standard one-stage factor VIII clotting-activity assays and Bethesda inhibitor assays based on measurement of the activated partial-thromboplastin time were performed (Automated APTT reagent, Organon Teknika) on an MDA instrument with the use of a standard curve prepared with a normal human pooled plasma calibration standard (Precision Biologic), which was calibrated against a World Health Organization standard. Abnormal controls (Dade and Behring) and normal controls (Precision Biologic), both derived from human plasma, were used to validate the assays. Serum samples from the patients were analyzed for antibodies against factor VIII with two specific enzyme-linked immunosorbent assays. Full-length human factor VIII derived from Chinese-hamster-ovary cells (Recombinate, Hyland–Baxter) or human plasma-derived factor VIII (Hemofil-M, Hyland–Baxter) was used to coat the wells of microtiter plates before the addition of patients' serum. The enzyme-linked immunosorbent assay was developed with a horseradish peroxidase–conjugated goat antihuman antibody. The assay was validated with the use of normal human plasma and abnormal human plasma containing various amounts of factor VIII antibodies (George King Bio-Medical). All specialized blood-coagulation testing was performed at Esoterix Coagulation Laboratories with use of frozen plasma or serum samples that were collected from the patients at all scheduled visits; however, at month 9 only factor VIII activity was tested, and only in Patients 5 and 6.

RESULTS

Characteristics of the Patients and Factor VIII Production by the Implanted Fibroblasts

Table 1 shows the characteristics of the six patients. As shown in Table 2, Patients 1, 2, and 3 each received 100 million cells, and Patients 4, 5, and 6 each received 400 million cells. The total factor VIII production of each of the clones that was implanted is also shown.

Safety

The skin biopsy was well tolerated by all six patients, and there were no episodes of bleeding or infectious

TABLE 1. CHARACTERISTICS OF THE SIX PATIENTS.*

CHARACTERISTIC	VALUE
Age (yr)	
Mean	46
Range	20–72
Weight (kg)	
Mean	70
Range	50–91
Pretreatment factor VIII activity <0.8% of normal (no. of patients)	6
Viral exposure (no. of patients)†	
Human immunodeficiency virus	4
Hepatitis A virus	5
Hepatitis B virus	5
Hepatitis C virus	6

*All six patients were men.

†Viral exposure was determined at the time of enrollment by testing for the presence of antibodies to the viruses listed.

TABLE 2. TOTAL FACTOR VIII PRODUCTION BY IMPLANTED AUTOLOGOUS FIBROBLASTS.*

PATIENT NO.	FACTOR VIII PRODUCTION BY HARVESTED CELLS†	NO. OF CELLS IMPLANTED	TOTAL FACTOR VIII PRODUCTION BY IMPLANTED CELLS‡
	IU/10⁶ cells/day		IU/kg/day
1	0.8	100×10⁶	1.3
2	4.9	100×10⁶	5.4
3	1.9	100×10⁶	3.8
4	1.8	400×10⁶	10.4
5	1.6	400×10⁶	8.4
6	6.7	400×10⁶	36.0

*The conditioned medium of each fibroblast clone was replaced with fresh medium 24 hours before it was assayed for factor VIII expression levels by a human factor VIII enzyme-linked immunosorbent assay.

†The production of factor VIII at the time of cell harvest, before implantation, is shown.

‡The total factor VIII production of each implanted clone is shown, normalized for the weight of each patient.

complications. Laparoscopic implantation of the fibroblasts was also well tolerated. The mean (±SD) duration of surgery was 65±7 minutes. The mean time required for the injection of the fibroblasts was 15±3 minutes. There were no complications related to anesthesia. After the procedure, all six patients had minor abdominal discomfort at the incision site. Other adverse events during the postoperative period were pain referred to the shoulder in five patients, abdominal ecchymoses in two, and low-grade postoperative fever (which did not require treatment) in one. There were no long-term complications associated with surgery.

All the Bethesda inhibitor assays and enzyme-linked immunosorbent assays for antibodies against factor VIII were negative. Preliminary assays for cytotoxic T lymphocytes at selected times through month 12 in Patients 1, 2, and 3 and through month 6 in Patients 4, 5, and 6, in which the patients' lymphocytes were used as the effector cells and transfected autologous fibroblasts expressing B domain–deleted factor VIII were used as the target, were negative; this finding indicates that there was no cellular immune response to the cultured transfected fibroblasts.

One patient with a long-standing history of recurrent paroxysmal atrial tachycardia had two episodes of tachycardia, 7.5 months and 12 months after the implantation procedure. These two serious adverse events were classified as unrelated to the use of the genetically modified fibroblasts or the implantation. There were no other serious adverse events. All six patients had adverse events, most of which consisted of bleeding episodes or pain associated with bleeding. No clinically significant laboratory abnormalities related to the gene therapy were detected.

Clinical Effects

In addition to evaluating the safety of the use of the genetically engineered fibroblasts and of the surgical procedures (skin biopsy and laparoscopy), we appraised several subjective and objective measures of efficacy: bleeding events related to hemophilia, use of exogenous factor VIII for treatment before and during the study, and factor VIII activity levels. Figure 2 shows bleeding events and the use of exogenous factor VIII in Patients 1, 3, and 6, all of whom maintained diaries of bleeding and factor VIII use before participation in the study. Patients 2, 4, and 5 did not keep such diaries before entering the study.

In Patient 1, the frequency of bleeding and the amount of exogenous factor VIII used did not change after implantation of the transfected fibroblasts. This patient received the implant with the lowest level of factor VIII production in vitro of any of the implants (Table 2), and no clinically important increase in factor VIII activity was detected in his plasma after therapy (Table 3). Beginning 10 weeks after the implantation of transfected fibroblasts, Patient 3 had no bleeding, and he did not use factor VIII for the treatment of new bleeding for the next 3 months. At week 18, nearly eight weeks after the most recent infusion of factor VIII, his plasma factor VIII activity had increased from less than 0.4 percent of normal to 2.0 percent of normal (Table 3). There were injury-related bleeding episodes, but he reported no spontaneous bleeding. There were several prolonged intervals without bleeding or infusions of factor VIII (Fig. 2). Additional measurements of factor VIII activity showed an activity level of 1.0 percent of normal at 6 months and a decline to less than 0.5 percent at 12 months.

Patient 6 received the fibroblast clone that produced the largest amount of factor VIII per day in vitro of any of the clones (Table 2). A decreased frequency of bleeding became evident one month after the transfected fibroblasts were implanted (Fig. 2). During periods when there was no bleeding or use of exogenous factor VIII, numerous measurements of factor VIII activity showed a persistent increase in activity to a level above that observed at the initial evaluation (Table 3). Like Patient 3, he reported no spontaneous bleeding for approximately 10 months after the implantation (Fig. 2). However, there was intermittent injury-related hemorrhage. The frequency of bleeding in this patient increased and spontaneous bleeding recurred during month 12, at which time his level of factor VIII activity had declined to less than 0.5 percent of normal (Table 3).

Table 4 shows the average monthly use of factor VIII before and after cell implantation in all six patients. Treatment with infusions of exogenous factor VIII before enrollment in the study was documented by the patients' diaries (Patients 1, 3, and 6) or by pharmacy records (Patients 2, 4, and 5). Use of exogenous factor VIII by Patients 1 and 2 was unchanged after treatment. Use by Patients 3, 4, and 5 was moderately decreased. In Patient 6 there was a considerable reduction in the use of exogenous factor VIII after gene therapy; in the 20 months before therapy, his total use of factor VIII each month ranged from 12,339 to 30,621 U, whereas his use ranged from 2040 to 7098 U per month at months 4, 6, 7, 8, and 10 after gene therapy (Table 4).

A standard one-stage assay of factor VIII activity was used to detect changes in factor VIII activity over time (Table 3). Levels of factor VIII activity above pretreatment levels were repeatedly found after treatment in Patients 3, 4, 5, and 6. Patient 3 had a factor VIII activity level of 2.0 percent of normal at week 18 (eight weeks after his most recent factor VIII infusion). It was 1.0 percent of normal at month 6, nine days after a single infusion of factor VIII. These elevated levels were not the result of the use of exogenous factor VIII, because factor VIII activity was undetectable at other points closer in time to a preceding infusion (weeks 2, 6, 8, and 12). Patient 4 had factor VIII activity levels of 0.8 percent and 0.6 percent of normal at weeks 4 and 6, respectively. Although both

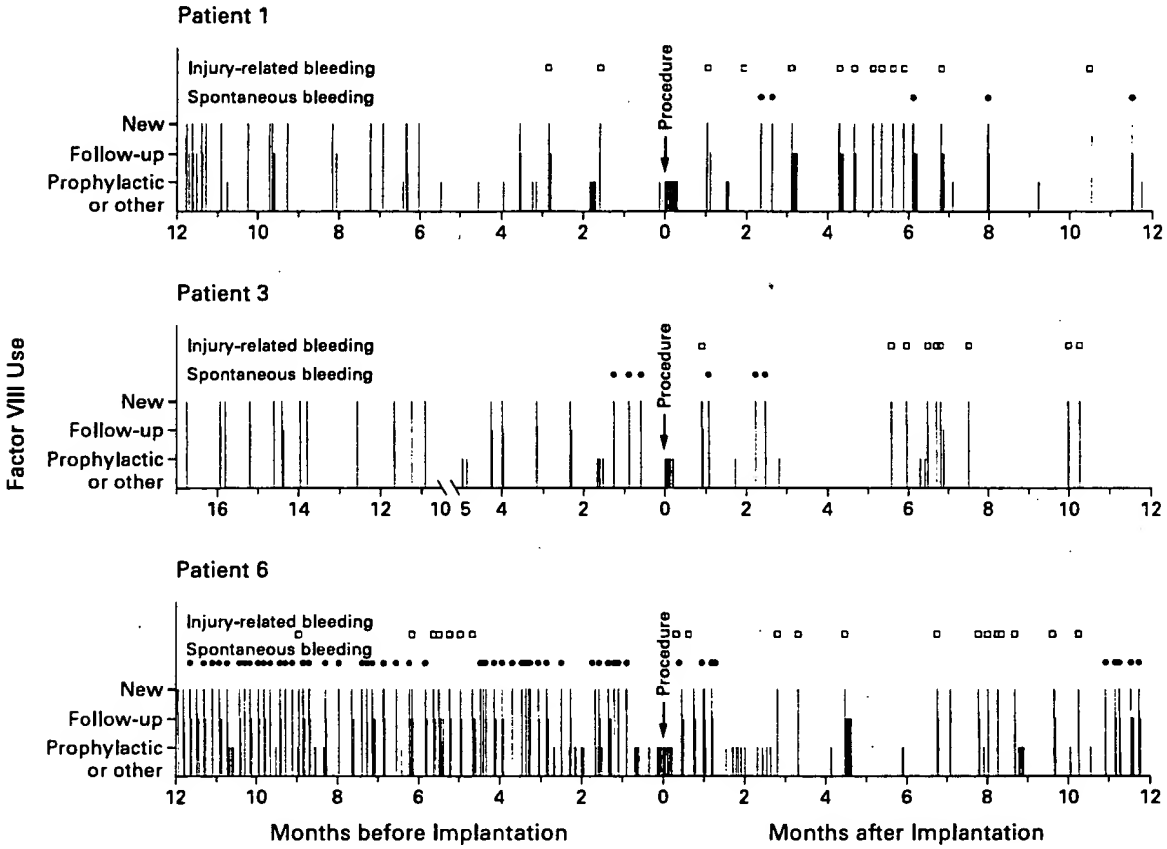

Figure 2. Bleeding Events and Use of Exogenous Factor VIII in Three of the Six Patients.

Bleeding events and use of exogenous factor VIII during the 12 months before and after the cell-implantation procedure (time 0) are shown. Each infusion of factor VIII is shown as a vertical line. The tallest lines correspond to infusions at the time of a new bleeding event. After enrollment in the study each of these new bleeding episodes was classified as injury related (open squares) or spontaneous (solid circles). Lines of intermediate height represent follow-up infusions to complete the treatment of a previous bleeding episode, and the shortest lines represent infusions for prophylaxis or other reasons, such as the surgical procedures in the study. In the chart for Patient 3, pretreatment data over a 5-month period (between 10 months and 5 months before the implantation) are omitted; during this period the patient used fixed-dose factor VIII as secondary prophylaxis, and neither the frequency of bleeding nor the use of factor VIII was representative of that at other times before entry into the study. For Patients 1 and 3, bleeding episodes were recorded but not classified until enrollment in the study 3.3 and 1.7 months, respectively, before the implantation procedure. For Patient 6, episodes that occurred 11.9, 11.8, 11.5, 7.7, and 2.3 months before and 1.0 and 7.1 months after the implantation procedure were inadvertently not classified. Monthly intervals are 30 days.

of these levels were above his initial level before the implantation (less than 0.4 percent of normal), it is unclear whether these small changes are clinically significant. Nevertheless, the appearance of measurable factor VIII coincided with decreased use of exogenous factor VIII. In Patient 5, several measurements of factor VIII activity between week 4 and month 6 after the implantation were above his initial level (which was undetectable). Although the change in the use of factor VIII was small in this patient, there was a general correlation between decreased use of factor VIII through month 10 and the duration of the elevation in factor VIII activity. Patient 6 had the highest measurable level of activity after gene therapy; at week 12, the activity was 4.0 percent of normal. At months 9 and 12, the activity in this patient had decreased to less than 0.5 percent of normal, and spontaneous bleeding recurred.

DISCUSSION

We studied six patients with severe hemophilia A who received autologous fibroblasts that carried the gene that encodes factor VIII. The implantation procedure was safe, and the cells have been well tolerated,

TABLE 3. LEVELS OF FACTOR VIII ACTIVITY.*

TIME POINT	PATIENT					
	1	2	3	4	5	6
	% of normal level					
Initial evaluation	<0.8	<0.8	<0.8	<0.4	0	0.5
Day before cell implantation	<0.8 (3 to <4 days)	<0.8	<0.4	<0.4	<0.4 (3 to <4 days)	4.0 (1 to <2 days)
Week 2	<0.8	<0.8	<0.4	<0.8	3.0 (1 to <2 days)	102 (<1 day)
Week 3	<0.8	<0.4	<0.8	<0.4	2.0 (3 to <4 days)	2.0†
Week 4	<0.8	<0.4	19.0 (<1 day)	0.8†	1.0†	3.0†
Week 6	<0.8	0.8 (3 to <4 days)	<0.4	0.6†	18.0 (<1 day)	1.0†
Week 8	<0.8	<0.4	<0.4	<0.5	1.0†	5.0 (1 to <2 days)
Week 12	<0.8	<0.4	<0.4	<0.4	1.0†	4.0†
Week 18	<0.4	<0.4	2.0†	<0.5	0.5†	1.0†
Month 6	<0.4	0.7	1.0†	<0.5	1.0†	2.0†
Month 9	—	—	—	—	1.0 (1 to <2 days)	<0.5
Month 12	0.5	<0.5	<0.5	0.5†	<0.5	<0.5

*Some measurements of factor VIII activity levels were most likely influenced by previous infusions of exogenous factor VIII. The interval between measurement and the most recent infusion of factor VIII is given in parentheses. Otherwise, the factor VIII activity level was determined at least five days after a previous factor VIII infusion. Factor VIII activity levels were measured at scheduled visits: weeks 2 and 3 (±1 day); weeks 4 and 6 (±2 days); weeks 8 and 12 (±4 days); and week 18 and months 6, 9, and 12 (±2 weeks). Dashes indicate that measurement of factor VIII activity was not performed.

†The factor VIII activity level is considered elevated above levels measured before implantation.

a finding that has continued in all patients during a second year of monitoring. In no patient did evidence of factor VIII inhibitor appear at any time during the study. This is important, because inhibitors have the potential to compromise the effectiveness of standard factor VIII–replacement therapy.[19]

The potential for clinical efficacy of this type of gene therapy is suggested by the increased levels of factor VIII activity in four of the six patients. Before they entered the study, the factor VIII activity levels in all six patients were less than 0.8 percent of normal — a fact that made it possible to detect relatively small increases in factor VIII in plasma after the implantation of the genetically engineered fibroblasts. There was a general correlation between these increases and clinical improvement, such as a decreased frequency of spontaneous-bleeding episodes or decreased use of exogenous factor VIII.

At the relatively low levels of factor VIII activity we observed, several factors may have influenced subjective measures of the clinical efficacy of the gene-therapy procedure we tested. One of these is the presence or absence of active joint disease: treatment of severe joint disease requires persistent, relatively high levels of factor VIII activity. The patients' investment in the therapeutic outcome of the experimental treatment may also have influenced subjective measures of efficacy that suggested therapeutic benefit. However, the correlation between objective and subjective signs of improvement indicates that a prolonged placebo effect was unlikely.

The minimal levels of factor VIII that are required to protect against spontaneous or post-traumatic bleeding are unknown. Protection against bleeding related to trauma or surgery may require high factor VIII levels, in the range of 30 to 100 percent of normal, depending on the nature of the injury.[20] In contrast, the clinical features that distinguish between severe and moderate hemophilia suggest that factor VIII activity levels as low as 1.0 to 2.0 percent of normal can protect against spontaneous bleeding. A recent study of prophylactic treatment in patients with severe hemophilia indicated that even levels below 1.0 percent of normal may be sufficient.[21] For these reasons, the clinical responses we observed in our patients, who had low plasma levels of factor VIII, are encouraging.

The nonviral ex vivo gene-therapy system we used has several potential advantages over gene therapy based on viral vectors. Patients received a homogeneous clonal population of cells containing a single genetic modification, thereby minimizing the risk of insertional mutagenesis that might occur with viral vectors. In addition, viral vectors can evoke immune responses that may attenuate the effectiveness of sub-

TABLE 4. MONTHLY USE OF EXOGENOUS FACTOR VIII.*

PATIENT NO.	REASON FOR FACTOR VIII TREATMENT	BEFORE CELL IMPLANTATION†	MONTHS AFTER CELL IMPLANTATION											
			1	2	3	4	5	6	7	8	9	10	11	12
			IU of factor VIII											
1	Spontaneous bleeding		0	0	5,153	0	0	0	11,766	2,220	2,220	0	0	4,480
	Injury-related bleeding		0	4,100	0	10,024	15,326	8,320	6,426	0	0	0	2,220	0
	Prophylaxis or other		14,431	4,160	0	0	0	0	0	2,220	0	2,220	0	2,240
	Total	5,904±4,617	14,431	8,260	5,153	10,024	15,326	8,320	18,192	4,440	2,220	2,220	2,220	6,720
2	Spontaneous bleeding		2,000	2,000	0	0	2,060	2,060	6,140	6,180	0	0	4,120	2,060
	Injury-related bleeding		0	0	4,000	4,120	2,020	0	0	0	1,010	0	0	2,020
	Prophylaxis or other		28,860	0	0	2,020	0	0	0	0	0	0	0	0
	Total	4,798	30,860	2,000	4,000	6,140	4,080	2,060	6,140	6,180	1,010	0	4,120	4,080
3	Spontaneous bleeding		0	2,080	2,625	0	0	0	0	0	0	0	0	0
	Injury-related bleeding		2,865	0	0	0	0	4,300	6,550	2,200	0	0	3,825	0
	Prophylaxis or other		15,283	1,040	1,050	0	0	0	2,200	0	0	0	0	0
	Total	3,726±1,799	18,148	3,120	3,675	0	0	4,300	8,750	2,200	0	0	3,825	0
4	Spontaneous bleeding		3,264	2,736	5,510	7,996	3,831	3,360	8,768	2,992	20,218	10,282	24,542	17,535
	Injury-related bleeding		0	0	0	0	0	0	0	0	0	0	0	0
	Prophylaxis or other		15,833	0	0	0	0	0	0	0	5,600	2,510	0	2,721
	Total	16,303	19,097	2,736	5,510	7,996	3,831	3,360	8,768	2,992	25,818	12,792	24,542	20,256
5	Spontaneous bleeding		5,250	6,940	11,068	13,024	8,320	10,175	4,625	8,495	9,090	10,100	13,100	20,928
	Injury-related bleeding		2,100	2,100	1,956	10,980	2,080	0	2,775	1,850	0	0	0	2,160
	Prophylaxis or other		28,450	0	1,956	0	0	0	0	925	3,030	1,010	2,988	1,992
	Total	14,630	35,800	9,040	14,980	24,004	10,400	10,175	7,400	11,270	12,120	11,110	16,088	25,080
6	Spontaneous bleeding		9,031	4,240	0	0	0	0	0	0	0	0	2,196	23,564
	Injury-related bleeding		4,925	0	2,316	2,040	22,340	0	5,298	3,532	6,242	5,490	2,196	0
	Prophylaxis or other		23,019	15,040	9,960	0	1,766	4,420	0	3,566	18,174	0	5,728	0
	Total	19,052±5,133	36,975	19,280	12,276	2,040	24,106	4,420	5,298	7,098	24,416	5,490	10,120	23,564

*The patients recorded their use of factor VIII on diary forms and categorized each infusion according to the reason for administration. When a bleeding episode was treated with more than a single infusion of factor VIII, all the follow-up infusions were classified in the same category as the initial infusion. The category "prophylaxis or other" includes the use of factor VIII for surgical procedures such as the skin biopsy, the cell-implantation procedure, or other procedures, such as dental work.

†Data were available for the following intervals before treatment: Patient 1, 13 months; Patient 2, 14 months; Patient 3, 28 months (except the period from 10 months through 5 months before implantation, when he took a fixed-dose factor VIII as secondary prophylaxis); Patient 4, 14 months; Patient 5, 24 months; and Patient 6, 20 months. All monthly intervals were 30 days. Plus–minus values are means ±SD and represent monthly averages for data taken from patient diaries. When average monthly use was determined from pharmacy records, the SD is omitted.

sequent treatments.[22] Viral vectors may become infectious by mutation or other mechanisms or may modify the patient's germ line, but these risks are obviated with the use of the nonviral approach. Despite these advantages, our system has noteworthy disadvantages. The implantation procedure is moderately invasive, and the factor VIII–producing autologous fibroblasts must be prepared individually for each patient. Fortunately, there is no technological obstacle to the production of such patient-specific fibroblasts, so the procedure could be feasible if the therapeutic benefit of this approach can be proved in subsequent studies.

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Drs. Treco and Selden are employees of and own stock in Transkaryotic Therapies.

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APPENDIX

Other members of the Factor VIII Transkaryotic Therapy Study Group were as follows (asterisks indicate a financial interest in Transkaryotic Therapies): J.D. Levine, J. Proper, and B. Furie* (Beth Israel Deaconess Medical Center and Harvard Medical School, Boston) and V.A. Roman,* Z.M. Sabine,* C.W. Phillips,* A.M. Zuliani,* N.A. Savioli,* D. Fisher,* J. Harrington,* M. Borowski,* M.W. Heartlein,* J.C. Lamsa,* E.M. Morrel,* and K.C. Gunter* (Transkaryotic Therapies, Cambridge, Mass.).

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EXHIBIT D

Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion

Jerry S. Powell, Margaret V. Ragni, Gilbert C. White II, Jeanne M. Lusher, Carol Hillman-Wiseman, Tom E. Moon, Veronica Cole, Sandhya Ramanathan-Girish, Holger Roehl, Nancy Sajjadi, Douglas J. Jolly, and Deborah Hurst

In a phase 1 dose escalation study, 13 subjects with hemophilia A received by peripheral intravenous infusion a retroviral vector carrying a B-domain–deleted human factor VIII (hFVIII) gene. Infusions were well tolerated. Tests for replication competent retrovirus have been negative. Polymerase chain reaction (PCR) analyses demonstrate the persistence of vector gene sequences in peripheral blood mononuclear cells in 3 of 3 subjects tested. Factor VIII was measured in serial samples using both a one-stage clotting assay and a chromogenic assay. While no subject had sustained FVIII increases, 9 subjects had FVIII higher than 1% on at least 2 occasions 5 or more days after infusion of exogenous FVIII, with isolated levels that ranged from 2.3% to 19%. Pharmacokinetic parameters of exogenous FVIII infused into subjects 13 weeks after vector infusion showed an increased half-life ($T_{1/2}$; $P < .02$) and area under the curve (AUC, $P < .04$) compared with pre-study values. Bleeding frequency decreased in 5 subjects compared with historical rates. These results demonstrate that this retroviral vector (hFVIII(V)) is safe and, in some subjects, persists more than a year in peripheral blood mononuclear cells, with measurable factor VIII levels and with increased available FVIII activity (increased $T_{1/2}$ and AUC) after infusion of exogenous FVIII concentrate. (Blood. 2003;102:2038-2045)

Introduction

Hemophilia A is an X-linked bleeding disorder caused by a deficiency or abnormality of factor VIII (FVIII), a necessary cofactor for the generation of thrombin. It is the most common inheritable coagulation protein deficiency, with an incidence of approximately 1 in 10 000 males.[1] Individuals with severe hemophilia suffer episodes of spontaneous bleeding in joints and soft tissue, including intracranial hemorrhage. Recurrent joint bleeding leads to characteristic chronic debilitating hemophilic arthropathy. The development of joint disease and other complications of hemorrhage results in absences from school and work, and chronic pain, and often leads to disability.

Hemophilia is treated by replacement of the missing clotting factor using intravenous infusion of FVIII protein concentrates. Such infusions must be administered frequently for hemostasis after serious bleeding episodes because the half-life of FVIII protein is only 8 to 12 hours. To sustain prophylactic FVIII levels, infusions are administered every other day. While current therapeutic products for hemophilia A, including plasma-derived factor VIII concentrates and recombinant FVIII products, have significantly lowered the risk of viral transmission and offer reliable prophylactic and therapeutic efficacy,[2] there are lingering concerns about potential viral contamination and transmission of other infectious agents. Furthermore, the concentrates are extremely expensive and require frequent intravenous administration, sometimes necessitating placement of venous access devices. For these and other reasons, gene therapy for hemophilia has been of considerable interest.

FVIII deficiency is a promising genetic disease to target for gene therapy for a number of reasons.[3-6] Delivery and expression of the normal FVIII gene in any tissue with vascular exposure that leads to even low levels of expression of FVIII protein would be expected to prevent spontaneous bleeding. In addition, it is likely that neither tissue-specific expression nor exogenous control of gene expression is required, making this approach relatively straightforward and achievable with current gene transfer methods. The gene for FVIII is well characterized and there are excellent preclinical mouse and dog models of hemophilia. Successful FVIII gene therapy could be expected to reduce health care expenditures as well as improve the quality of life for these individuals.[7,8]

In this report, we describe the results of a phase 1, single-treatment, dose-escalation study of gene therapy using a retroviral vector in subjects with hemophilia A. The retroviral vector (hFVIII(V)) was based on a type C retrovirus, Moloney murine leukemia virus (MoMLV), that was rendered replication deficient and carried a B-domain–deleted gene for human factor VIII (hFVIII). The vector has been extensively modified to carry no genes encoding viral proteins, and to minimize or eliminate regions of homology between vector and producer cell sequences in order

From the University of California at Davis, Sacramento; the University of Pittsburgh (UP), General Clinical Research Center, PA; the University of North Carolina (UNC), Chapel Hill; Wayne State University, Detroit, MI; Chiron, Emeryville, CA; the Chiron Center for Gene Therapy, San Diego, CA; Cell Genesys, San Diego, CA; Biomedica, San Diego, CA; and Sajjadi Associates, Encinitas, CA.

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Reprints: Jerry S. Powell, University of California at Davis, Division of Hematology and Oncology, Suite 3016, University of California Davis Cancer Center, 4501 X St, Sacramento, CA 95817; e-mail: jspowell@ucdavis.edu.

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to reduce the chance of a recombination event during production. Safety of the vector was supported by preclinical studies in mice, rabbits, and normal and hemophilia A–infected dogs.[9-13] The same proprietary vector system had been used previously to produce vectors carrying genes for clinical applications in cancer and human immunodeficiency virus (HIV) disease, and these preparations have been directly administered to more than 300 subjects without safety problems in phase 1 and phase 2 clinical trials.[14-17] However, this study was the first using intravenous administration, and, therefore, safety and tolerability were carefully monitored. The results of the trial demonstrate the safety of this retroviral vector in human subjects with hemophilia A.

Patients, materials, and methods

Study design

This phase 1 clinical trial was designed as a multicenter, intersubject dose-escalation, single-treatment, open-label trial in adult male subjects with severe hemophilia A. We planned to enroll between 9 and 20 subjects, and to follow them closely for an initial period of 13 weeks (phase 1) when they were predicted to show first appearance of measurable FVIII activity and when they would be monitored for early adverse events related to the infusion of the retroviral construct. Subsequently, they were to be seen at less frequent intervals through 53 weeks after treatment (phase 1 extension). This report presents the results through the first 53 weeks of study. Subsequently, these subjects have been asked to enroll into a long-term safety surveillance program in accord with FDA guidelines for the follow-up of individuals who have received retroviral constructs.

Study subjects

All subjects gave signed informed consent after the study design and protocol were approved by the individual institutional review boards (IRBs; listed in "Acknowledgments") of the participating centers. The study protocol was submitted and approved, as part of an investigational new drug (IND) application, by the Center for Biologics Evaluation and Research of the United States Food and Drug Administration and by the Recombinant DNA Advisory Committee of the Office of Biotechnology Activities of the National Institutes of Heath.

Inclusion criteria included adult males (age, > 18 years) with severe hemophilia A (< 1% FVIII, measured following ≥ 5 days without FVIII treatment) who had received 100 or more treatments with FVIII concentrates in the past, who had no history of FVIII inhibitor, and whose current inhibitor titer was less than 0.6 Bethesda units (BU). For each potential subject, a pharmacokinetic study was conducted by measuring FVIII activity following infusion of 50 IU/kg of the subject's usual FVIII product. The FVIII activity assay at 6 hours had to be higher than 30% of the 10-minute peak, and FVIII activity had to be detectable between 24 and 36 hours after FVIII treatment. While participating in the study, the subjects had to be willing and able to use "on demand" treatment only (for active bleeding episodes), rather than prophylactic treatment.

Subjects who were seropositive for HIV could be enrolled in this study if their CD4 counts were higher than 300 cells/mm^3 and they were not being treated concurrently with reverse-transcriptase inhibitors. Subjects had to agree to use a barrier method of contraception throughout the study (53 weeks), and, if vector was detected in the semen, until 3 consecutive monthly semen specimens showed no detectable vector. All subjects were seropositive for hepatitis C antibody; no specified viral RNA level or liver histology by biopsy was required for participation in the study.

Exclusion criteria included any significant cardiac, pulmonary, endocrine, neurologic, or hematologic condition, other than hemophilia A; alanine transaminase (ALT) value 5 times or more than normal; abnormal laboratory values for albumin, bilirubin, or prothrombin time (PT); treatment with interferon or ribavirin within 3 months of enrollment; prior history of allergic reactions to factor VIII or prophylactic medication required to prevent such reactions; or therapy with any non-FVIII investigational agent within 30 days of enrollment. Those individuals who had received investigational FVIII within 30 days were required to have had at least 50 days of exposure to that preparation and no drug-related adverse events.

Viral vector construct

The viral vector construct, hFVIII(V) (Chiron, Emeryville, CA), in this trial consisted of a retroviral vector, based on the Moloney murine leukemia virus (MoMLV), carrying a B-domain–deleted gene for human factor VIII (hFVIII). The B-domain–deleted form of the FVIII gene was used because vectors carrying this gene consistently yielded higher titers and higher levels of protein expression per gene copy number compared with the full-length gene (Sheridan et al, unpublished data, May 1999). Experience with a commercially available recombinant form of FVIII produced from cells lacking the B-domain has shown that this alteration does not affect hemostatic ability or immunogenicity of the FVIII molecule.[18,19] The vector has an amphotrophic envelope and is made in a human packaging cell line. The vector is introduced into the packaging cell line by transduction of a vesicular stomatitis virus G protein (VSVg)–pseudotyped particle made by transient transfection. This procedure produces a high titer vector that is resistant to inactivation by human complement.[20,21] The vector[21] carries a single gene (FVIII) and expression is driven from the promoter in the Moloney 5′ long terminal repeat (LTR). Similarly, the 3′ polyadenylation site is provided by the 3′ LTR. The vector carries no genes for viral proteins, and retroviral sequences that remain were modified to minimize or eliminate regions of homology between vector and producer cell sequences in order to reduce the chance of a recombination event during production of the vector. The vector (hFVIII(V)) was harvested from the supernatant of cells grown in the CellCube (Corning Costar, Cambridge, MA) as described and was purified using ion exchange and column chromatography.[22] The final product was formulated with lactose and phosphate-buffered saline for intravenous infusion. Vector titer was determined by adding a dilution series of the vector to human fibrosarcoma cells (HT 1080) and performing limiting dilution polymerase chain reaction (PCR) analysis to determine the number of DNA vector genomes (provector copies) present in each member of the original dilution series. One provector copy was termed a transducing unit (TU).

Extensive testing of this retroviral construct in animals demonstrated safety and potential efficacy.[9-12,23] The highest FVIII levels were obtained in adult animals when total doses were divided and administered over consecutive days. At doses similar to those planned for this phase 1 clinical trial, rabbits showed reproducible production of hFVIII protein and sustained expression, for as long as the rabbits were followed, of concentrations of FVIII protein of 30 to 40 ng/mL (which would be equivalent to 15%-20% FVIII activity in humans). There were 2 studies conducted in hemophilic dogs. In one study, a shortening of the whole blood clotting time (WBCT) from longer than 40 minutes to 12 to 22 minutes was observed between days 4 and 14 in 6 of 7 treated animals (normal control dogs had WBCT values of 6-10 minutes).[23] In addition, between days 4 and 14 (the time the dogs showed shortening of the WBCT), hFVIII concentrations, measured by enzyme-linked immunosorbent assay (ELISA), increased to a range between 25 to 90 ng/mL. Immune complexes between vector-derived hFVIII and canine immunoglobulin G (IgG) were detected initially at low levels, coinciding with the period of partial WBCT correction. After day 14, the amounts of immune complex formation increased rapidly, coinciding with the period when most of the dogs showed return of WBCTs to pretreatment levels. In 2 animals, however, WBCT remained shortened (approximately 21 minutes) 6 to 12 months after treatment, again coinciding with decreasing amounts of canine IgG:hFVIII complexes. In a second set of experiments the vector was delivered at 3 dose levels in 3 dogs per dose level.[12,13] Significant concentrations of human factor FVIII were observed in 1 of 3 dogs at each dose level and reduced bleeding and shortened partial prothrombin time (PTT) were observed in all 9 dogs. The dog experiments were complicated by the appearance of dog antihuman FVIII antibodies. In addition to these studies, extensive toxicology studies performed in rabbits and mice established that dose ranges associated with potentially therapeutic FVIII levels in plasma

could be administered without significant toxicity. Detection of vector-specific sequences in tissues, primarily liver and spleen, was not associated with histopathologic changes.

Coagulation assays

Coagulation tests, including FVIII levels, inhibitor assays, prothrombin time, partial thromboplastin time, and chromogenic assays, were performed in a central reference laboratory at the Special Coagulation Laboratory, Children's Hospital of Michigan. All samples had code numbers only, and all assays were performed blinded. Assays were performed on frozen samples that had been stored at −80°C and thawed once in a 37°C waterbath. FVIII activity was measured both by a one-stage coagulation assay and by a chromogenic assay (Coatest; Chromogenix, Milan, Italy).[24] Both techniques were used because B-domain–deleted FVIII has been reported to result in higher plasma FVIII activity values when measured by chromogenic assay than by coagulant assay.[25] The usual lower limit of detection for the FVIII coagulant activity assays in this reference laboratory was 0.7%; however, after recalibrating the assay using additional dilutions and control curves in order to modify the assay, levels as low as 0.2% could be measured reproducibly using the one-stage assay, and as low as 0.3% using the chromogenic assay. The standard curve was established using Biopool human reference plasma (HRP) standardized against WHO standard. A B-domain–deleted factor VIII concentrate was not used to standardize assays. In recalibrating the FVIII assays the plasmas were diluted with buffer. The coefficients of variance (CVs) of the FVIII assays were 1.87% with George King B-Fact, 1.99% with George King severe hemophilia A, 5.2% with mild hemophilia A (all within runs), and 6.5% with Biopool HRP, and 6.2% with Biopool Abnormal (the latter 2 between runs). The CV for the chromogenic FVIII assay with George King Fact was 3.1% within runs. FVIII protein was measured using an enzyme immunoassay (Immuno).[26] FVIII inhibitor antibodies were measured using a Nijmegen-modified Bethesda assay.[27,28] The CVs of the inhibitor assays were 4.6% within a run and 7.7% between runs.

Laboratory monitoring

Baseline clinical history, physical examinations, and laboratory testing, including complete blood counts (CBCs), urinalysis, serum chemistry panels, testing for HIV and, if positive, viral load and CD4 counts, and testing for hepatitis C antibody and viral load were performed prior to and then following treatment with the FVIII construct, hFVIII(V). Safety assessments included FVIII inhibitor assays; adverse events; physical examination; standard clinical laboratory tests (complete blood count [CBC] with differential, serum chemistries [albumin, alkaline phosphatase, alanine transaminase, amylase, aspartate aminotransaminase, blood urea nitrogen, calcium, cholesterol, creatinine, direct and total bilirubin, gamma-glutamyl transferase, total protein, triglycerides, uric acid, and globulin], activated partial thromboplastin time [aPTT], prothrombin time [PT], and urinalysis); and viral serologies for HIV, hepatitis C virus (HCV), hepatitis B surface antigen (HBsAg) and surface antibody (HBsAb), and hepatitis A IgG. CD4 counts and HIV RNA by PCR were assayed in HIV-positive subjects and HCV RNA by PCR was assayed in HCV-positive subjects. Antibodies to fetal bovine serum (FBS), a possible low level contaminant of the vector preparations, were also measured. In addition, testing was performed for replication competent retrovirus (RCR) by polymerase chain reaction (PCR) assay at baseline, and 6 and 12 months after treatment, and semen samples were analyzed from baseline, and at weeks 2, 6, 9, 11, 17, 29, and 53 for detectable vector sequences by PCR.[11] Unless otherwise noted, the sensitivity of individual PCR test wells was 1 copy per 1.5×10^5 diploid cell genomes.

Dose escalation and follow-up

Each subject received a single treatment of hFVIII(V), at 1 of 4 escalating levels, administered as equally divided doses on 3 consecutive days via peripheral venous access. The total dose levels were 2.8×10^7, 9.2×10^7, 2.2×10^8, 4.4×10^8, and 8.8×10^8 transducing units (TU)/kg. The total dose was divided and administered as 3 equal daily doses on 3 consecutive days via peripheral venous access.

The hFVIII(V) dose was infused each day via peripheral vein at the infusion rate of 2 mL per minute. There were 3 subjects who received the same dose and were monitored weekly for 7 weeks for FVIII inhibitor formation and safety parameters prior to treatment of additional subjects and dose escalation. The first subject receiving the next higher dose was monitored throughout the 3 infusions (72 hours) before 2 additional subjects were treated with that dose. FVIII protein expression was assessed with FVIII assays 3 times weekly. Bleeding episodes were treated with an infusion of the FVIII concentrate usually used by the subject. All FVIII concentrate infusions and bleeding episodes were reported in home diaries, which were collected weekly during the first 3 months of study and monthly throughout the rest of the year. Subjects were monitored for FVIII protein expression and safety, and, after 53 weeks, were to be enrolled in a lifelong surveillance protocol that requires annual visits and blood samples for RCR testing, according to appropriate current FDA guidelines.

Efficacy measurements included FVIII activity and protein expression and recording in home diaries any bleeding episodes and treatment for bleeding. Assays were also performed to detect antibodies to the viral vector. At week 13, a repeat 36-hour pharmacokinetic study was conducted by measuring FVIII activity concentrations following administration of 50 IU/kg of the subject's usual FVIII product.

Statistical considerations

All subjects who received hFVIII(V) were included in safety analyses. Continuous data are expressed as means ± SD and categoric data are expressed as proportions, unless otherwise specified. Descriptive statistics were used to summarize data. Pharmacokinetic parameters were calculated at baseline and at end of phase 1 (week 13 after infusion of vector) using standard noncompartmental methods and WinNonlin Professional Version 3.3 (Pharsight, Mountain View, CA). All calculations were performed prior to rounding. Differences in pharmacokinetic (PK) values for FVIII at the end of phase 1 compared with baseline were analyzed using a 2-sided paired Student t test. $P \leq .05$ was considered statistically significant.

Results

Subject characteristics

Enrolled and treated with hFVIII(V) were 13 subjects. Of these, 3 each received the first 4 doses (2.8×10^7, 9.2×10^7, 2.2×10^8, and 4.4×10^8 TU/kg) and 1 received the highest dose of 8.8×10^8 TU/kg. Of the subjects, 11 completed the entire study (53 weeks), and 2 withdrew for personal reasons (refused to comply with follow-up appointments) after 3 and 6 months, respectively. One subject was noncompliant with a number of study requirements prior to withdrawal and did not have pharmacokinetic studies completed. Thus, the pharmacokinetic studies, at baseline and at 13 weeks, included a total of 12 subjects.

The subjects who participated in the trial were representative of individuals with severe hemophilia A. Baseline characteristics summarizing severity and prior therapy for hemophilia A, and HIV and HCV status of the population are shown in Table 1. The 13 subjects had an average of 3.7 ± 2.6 spontaneous bleeding episodes per month, with various joints affected. The most common bleeding sites were elbows (92%), ankles (62%), knees (31%), shoulders (7.7%), and other (7.7%). The presence of a target joint was not an exclusion criterion. Of the subjects, 5 were seropositive for HIV and all 13 were positive for the hepatitis C virus.

Safety

All subjects were alive and in their usual states of health at the completion of the 53 weeks of study. The administration of vector

Table 1. Baseline and demographic characteristics

Characteristic	No.
Demographics	
Age, y (range)	37.5 ± 14.7 (18-55)
Weight, kg	83.1 ± 8.8
Race	12 white, 1 black
HIV and HCV status (%)	
HIV, no. with positive serology	5/13 (38)
HCV, no. with positive serology	13/13 (100)
FVIII treatment schedule (%)	
Prophylactic	1/13 (8)
On demand	12/13 (92)
FVIII therapy in past 12 months (range)	
Number of infusions	50 ± 40.5 (10-150)
FVIII usage, units	117 076 ± 52 000 (12-450 000)
Spontaneous bleeding episodes per month (range)	3.7 ± 2.6 (0.5-8.0)
Functional status (%)	
Work or attend school	8/13 (62)
Disabled, hemophilia related	5/13 (39)

hFVIII(V) via peripheral vein each day for 3 consecutive days was well-tolerated, with no complications associated with the infusions reported in any subject.

Safety monitoring studies indicated that the infusion of hFVIII(V) via peripheral vein at these doses in these subjects was safe and well tolerated. Adverse events considered related to hFVIII(V) were as follows: dizziness in 4, flushing in 4, increased blood pressure in 2, headache in 2, increased heart rate in 1, chest pain in 1, and positive semen PCR test for vector in 1. All were mild in severity, except for a moderately severe headache in one subject. Tests for replication competent retrovirus were performed at regular intervals during the trial and were consistently negative. There was no clinical exacerbation of pre-existing HIV- or HCV-associated disease. CD4 counts, and HIV RNA and HCV RNA titers showed no significant adverse trends. In addition, clinical parameters showed no trends that correlated with treatment or treatment doses.

No FVIII inhibitors were detected by either Bethesda assay or by FVIII recovery and pharmacokinetic studies. The HIV and HCV RNA data overall showed no trends of concern according to 2 independent reviewers. Clinical laboratory testing (CBC, chemistry, and urinalysis) also revealed no trends over the year of the study. Nonneutralizing antibodies to murine leukemia virus (MLV) were detected in all subjects after treatment. No increases over background were detected for antibodies to fetal bovine serum or to FVIII protein by ELISA.

Table 3. Detection of FVIII(V) vector in PBMCs

Dose group	Dose, TU/kg	Week 4	Week 13	Week 29	Week 53
1	2.8×10^7	12/12	12/12	10/12	4/8
2	9.2×10^7	4/4	7/8	11/12	8/8
3	2.2×10^8	ND	8/8	12/12	ND
4	4.4×10^8	4/4	12/12	4/4	ND
5	8.8×10^8	4/4	4/4	ND	ND

Numbers represent the number of PCR assays positive over the number of assays performed. When tested, each subject's sample was tested with 4 replicate assays. Thus at week 13 for dose group 1, for example, there were 12 replicate assays, 4 from each of 3 subjects in the group.

PBMC indicates peripheral blood mononuclear cell; ND, assay not performed.

The only study-related serious adverse event occurred at week 9 following hFVIII(V) infusion, with a semen test that resulted in a transient positive PCR signal using 2 primer sets for vector in 1 of 10 replicates (subject no. 01001) (Table 2). This subject had received dose 4 (4.4×10^8 TU). Further studies could not be performed to determine whether the signal was in the sperm or the white blood cell fraction of the semen because such studies require fresh specimens, and all 4 subsequent semen samples showed no evidence of vector. The samples during the remainder of the study showed no evidence of vector sequences. All semen samples at study completion were negative for vector.

Persistence of vector sequences

Results of studies to detect the presence of vector in peripheral blood mononuclear cells (PBMCs) of subjects are summarized in Table 3. These studies were performed as allowed by specimen availability, as they were not primary study end points. Through week 29, more than 90% of sample assays showed vector detectable in PBMCs. Of 16 assays on week-53 samples, 12 (75%) were positive for vector. The week-53 samples tested were from 4

Table 2. Analysis of semen for provector sequences

Subject ID no.	Week 0	2	6	9	11	17	29	53
02001	NT	Neg	NA, x 2	Neg	NA	Neg	Neg	NA
05001	NT	Neg	Neg	Neg	Neg	Neg	QNS	Off study (1)
05002	NT	Neg	QNS, x 2	Neg	QNS	QNS	Neg	Neg
03001	NT	Neg	Neg	Neg	Neg	NA	NA	Neg
05003	NT	Neg	Neg	NA	Neg	Neg	Neg	Neg
03002	NT	Neg*	NA	NA	Neg	Neg	Neg	Neg
02002	NT	Neg	Neg†	Neg	Wk 13: Neg	Wk 20: Neg	Neg	Neg
05004	NT	Neg	Neg	Neg	Neg*	Neg	Neg	Neg
03003	NT	NA	NA	NA	NA	NA	Off study (2)	NA
05005	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg
05006	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg
01001	NT	Neg	Neg	Pos^{TP}	Wk 12: 13, 15: Neg	Neg	Neg	Neg
05007	NT	Neg	Neg	NA	Neg	Neg	Neg	Neg

ID indicates identification; NT, not tested; pretreatment samples were tested only if provector sequences were detected in posttreatment samples; Neg, negative, NA, no sample available; subject unable or refused to obtain specimen; QNS, quantity of sample obtained was not sufficient for testing; (1), subject discontinued study for personal reasons; (2), subject was noncompliant, refused to provide samples, and withdrew from study; and Pos^{TP}, classified as a "transient positive" due to subsequent negative tests. This sample result of 1 positive of 10 replicates tested translates to a transduction frequency estimate of 1 in 3 million cells.

*Reduced limit of target sequence detection.

†One primer site showed one tenth positive, but second primer confirmed negative.

Table 4. Comparison of FVIII pharmacokinetic analyses at baseline and at end of phase 1 (week 13 after infusion of vector hFVIII(V))

	Dose, TU					
	2.8×10^7, n = 3	9.2×10^7, n = 3	2.2×10^8, n = 2*	4.4×10^8, n = 3	8.8×10^8, n = 1	Overall, n = 12
AUC†, (%FVIII/h)/(IU/kg) baseline	39 ± 21	40 ± 7	59	44 ± 9	24.7	43 ± 14
AUC, wk 13	47 ± 26	53 ± 3	63	39 ± 21	21.0	49 ± 17
% change from baseline‡	21.1	32.4	7.7	7.4	−14.9	15.4 (P = .04)§
$T_{1/2}$, h, baseline	13.8 ± 3.3	16.9 ± 1.5	18.5	15.9 ± 2.3	9.3	15.5 ± 3.1
$T_{1/2}$, h, wk 13	16.8 ± 5.2	23.5 ± 2.9	20.2	15.9 ± 0.6	11.7	18.4 ± 4.7
% change from baseline‡	21.9	38.9	9.3	−0.3	25.8	18.6 (P = .02)§
Cmax†, % FVIII/(IU/kg) baseline	2.1 ± 0.4	2.1 ± 0.3	2.3 ± 0.2	2.4 ± 0.6	2.3	2.2 ± 0.4
Cmax, wk 13	2.2 ± 0.5	2.5 ± 0.4	2.3 ± 0.3	2.6 ± 0.4	1.8	2.4 ± 0.4
% change from baseline‡	4.7	21.0	0.1	10.7	−20.9	7.1 (P = .18)§

*FVIII value at one hour for subject no. 02002 was treated as an outlier.
†Cmax and AUC were normalized for dose of FVIII administered in international units (IU) per kilogram (kg) of body weight.
‡Calculated as a percent increase from baseline. Calculations were performed prior to rounding off.
§P value (2-tail) calculated using a 2-sided paired t test.

subjects who received the 2 lowest doses of vector, including 1 subject at the lowest dose with positive assay results. Persistence of vector sequences even in one subject who received the lowest dose infused supports the conclusion that vector sequences are likely to be present in PBMCs at one year in subjects who received higher doses of the vector construct, hFVIII(V).

Pharmacokinetic studies

The results for comparison of the baseline and the second 36-hour pharmacokinetic analyses at week 13 after infusion of hFVIII(V) are shown in Table 4.

For the 13 subjects as a group, the area under the curve (AUC) of FVIII normalized for dose of FVIII administered (49 ± 17 vs 43 ± 14 [%FVIII/h]/[IU/kg]) and half-life ($T_{1/2}$) (18.4 ± 4.7 vs 15.5 ± 3.1 hours) was significantly greater for the pharmacokinetic study at week 13 following treatment compared with the baseline pharmacokinetic study. The percent change from baseline in the maximum concentration (Cmax), normalized for dose of FVIII administered for the pharmacokinetic study, showed a trend toward higher levels at week 13 following treatment with hFVIII(V). The FVIII recoveries for the pharmacokinetic analyses for the 3 subjects who received the second dose are shown in Figure 1.

Clinical effects

A summary of the treatment FVIII infusions per year before and after hFVIII(V) is represented in Figure 2. The prestudy number of infusions per year was obtained by history and/or clinic treatment records at the time of study enrollment, and the number after hFVIII(V) infusion was obtained prospectively from home diary records of treatments and bleeding episodes. There were 5 subjects who were treated with fewer infusions in the year after receiving the vector infusion than during the study. Subject no. 9 had been treated on prophylaxis 3 times weekly prior to enrollment to prevent recurrent bleeding in his knee. He agreed to forego prophylaxis when he enrolled in the study and bled infrequently after receiving the vector. Overall, however, no significant change in bleeding frequency was seen.

Small increases in basal concentrations of factor VIII were observed in some subjects. Of 12 subjects, 8 (66.7%) had detectable FVIII concentrations (> 1%) on 2 or more occasions (at least 5 days following an FVIII infusion) during the 53 weeks after treatment with hFVIII(V) ("responders") (Table 5). While 5 individuals showed repeated elevations, the others showed only 1 to 3 elevations. The first detectable FVIII activity responses occurred as early as 8 to 10 days after administration of hFVIII(V) and as late as more than 300 days. Chromogenic FVIII assays showed similar levels to the coagulant assays. FVIII antigen levels were similar to activity levels, indicating that the FVIII protein was functional.

There was no correlation between time to first detectable FVIII activity response and dose of hFVIII(V) administered. In addition, no correlation was seen between FVIII activity responses and the individual pharmacokinetic results for incremental recovery of factor VIII activity, AUC, or the half-life of infused factor VIII. During the year following administration of hFVIII(V), 5 subjects

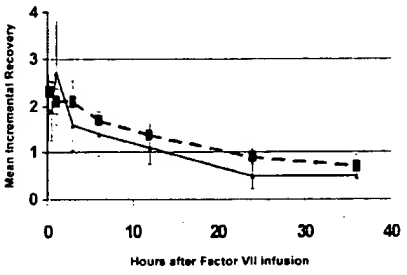

Figure 1. Mean FVIII recovery at week 13 is increased in subjects compared with mean FVIII recovery before hFVIII(V). Data shown are from the 3 subjects who received hFVIII(V) dose no. 2 (9.2×10^7 TU). Dashed line indicates FVIII recovery at week 13. Solid line indicates FVIII recovery at baseline pharmacokinetic study. FVIII incremental recovery is calculated as the percentage FVIII measured in plasma per unit of FVIII concentrate infused per kilogram body weight.

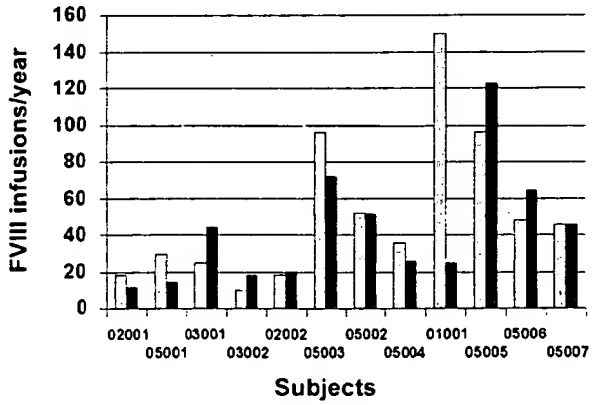

Figure 2. FVIII infusions per year before and after administration of hFVIII(V). The prestudy number of FVIII infusions per year was obtained by history at enrollment; the number after vector infusion was obtained prospectively from home diary records. No data are available for one subject. Hatched bar indicates before hFVIII(V); solid bar, after hFVIII(V).

Table 5. FVIII activity responses after hFVIII(V) administration

Dose and subject ID no.	No. of FVIII values 1% or higher/no. of observations	Study days	FVIII activity, %*	Infusions after hFVIII(V)†
Dose 1				
02001	2/23	113, 315	1.0, 1.8	Fewer
05001	1/8	93	6.6	Fewer
05002	10/22	10-297	1.0-3.0	No change
Dose 2				
03001	1/35	32	1.7	More
03002	14/38	19-159	1.0-1.3	More
05003	5/26	9-237	1.0-6.2	Fewer
Dose 3				
02002	2/6	302, 342	19, 1.1	More
03003	NA‡		—	
05004	5/15	8-211	1.1-2.6	Fewer
Dose 4				
01001	0/15	—	—	Fewer
05005	2/13	33, 44	1.0, 1.4	More
05006	4/11	40-317	1.1-4.4	More
Dose 5				
05007	2/37	11, 56	1.4, 2.1	No change

NA indicates not available.

*Reproducibility in the central laboratory, performed repeatedly on plasma samples from severe hemophilia A subjects, was X = 0.40, SD = 0.008, and CV = 1.99%.

†Comparison of number of treatments with factor VIII during the year prior to administration of hFVIII(V) with the number of treatments during the year after administration. Subject no. 05004 was receiving prophylaxis 3 times per week prior to entry on study.

‡Subject dropped out of the study for personal reasons and was noncompliant with FVIII assays and treatment records.

reported decreased use of factor VIII infusions. This apparent clinical response of less need for treatment did not correlate with administered dose of hFVIII(V) or with improved pharmacokinetic parameters. None of the doses tested resulted in FVIII concentrations higher than 7% in at least 75% of blood samples over a 12-week period in any of the subjects, one of the original goals of the trial based on human FVIII protein levels achieved in rabbits.

Discussion

This phase 1, multicenter, dose-escalation, FVIII gene therapy trial in adult hemophilia A patients used for the first time intravenous peripheral vein infusion of a retroviral-based vector construct. The results of this study demonstrate the clinical and biologic safety for this approach using hFVIII(V), a Moloney murine leukemia virus–based vector containing the gene for B-domain–deleted human factor VIII. The findings confirm the safety profiles seen in the animal studies using this vector construct. The vector was well tolerated and no adverse effects were detected after a year's follow-up. There were small clinical benefits, in some subjects, suggesting that further testing of this vector using alternative dose schedules to optimize FVIII protein expression is feasible and warranted.

Integration of hFVIII(V) into PBMCs was demonstrated by PCR in the majority (> 80%) of all specimens tested prior to week 53 and in 50% of the specimens tested at 53 weeks. Although vector could be detected in the majority of PBMC specimens, FVIII activity unrelated to exogenous treatment was low and transient. We suspect that, while the amount of protein was insufficient to cause a sustained measurable increase in the peripheral blood, enough vector-generated FVIII protein was produced to result in these borderline, intermittently measurable levels. Previous studies using retroviral vector transduction of autologous fibroblasts in large animals have reported long-term persistence of the vector sequences but decreased expression of the transgene product.[29-31] The mechanisms involved in loss of transgene expression despite persistence of vector sequences in those studies, as well as in the current human trial, have not been elucidated.

Of 12 subjects who were on study for at least 3 months, 9 had detectable FVIII activity (> 1%) on 2 or more occasions (range of maximum levels, 1.1%-19%). These FVIII levels were all measured at least 5 days after the last infusion of exogenous FVIII, according to infusion records from home diaries. The home diaries were collected weekly during the first 3 months of study and monthly throughout the first year in order to encourage timely and accurate reporting of home treatment. Nonetheless, it is possible that treatment records were not always completely accurate and that at least some FVIII levels measured could have been the result of residual exogenous FVIII from an unreported infusion. Although the FVIII levels were typically low and often transient, some responders reported fewer bleeding episodes, fewer courses of treatment for bleeding, and fewer units of FVIII product treatment while on-study compared with the prior year's treatment. However, none of these apparent trends for responders was statistically significant compared with nonresponders.

Alteration of the vector to optimize transcription of the integrated gene may increase the yield of FVIII protein in future studies. Another possible approach would be to administer repeated hFVIII(V) doses separated by longer intervals, in order to increase the likelihood of exposure to additional replicating cells, since retroviral (nonlentivirus) vectors preferentially enter the cell nucleus during mitotic cell division.[32,33] Repeated doses over time may result in a higher number of cells being transduced, if more or different cell populations are in cell cycle at the time of vector infusion, but this hypothesis requires further study for confirmation.

There was no evidence for a dose-response relationship between dose of vector administered and FVIII levels subsequently, suggesting that increasing the amount of hFVIII(V) used in a single dose would not be particularly useful, although the lack of a dose response may simply indicate that the current doses are near a therapeutic threshold. At least 2 potential avenues for further study may be promising. Since retroviral constructs preferentially transduce dividing cells, several laboratories are studying the role for growth factors to stimulate transiently the number of cells in cell cycle.[34,35] Alternatively, more frequent administration of hFVIII(V) (eg, one day each 2-3 weeks) might also allow additional newly cycling cells to be transduced.

Although clinical efficacy was not a primary objective in this phase 1 trial, the historical data on past bleeding frequency from hemophilia center records were compared with frequency measured prospectively after vector administration. Methods of past record keeping varied among sites and individual subjects. More uniform and reliable data could be collected in future studies by incorporating a run-in period prior to vector administration to collect baseline bleeding frequency. However, even with the best record keeping, treatment frequency in adults is not an ideal efficacy parameter because individuals with pre-existing musculoskeletal damage are highly susceptible to bleeding and would be unlikely to show significant decreases in frequency due to low levels of circulating FVIII. In contrast, young children or other individuals with normal joints and muscles have shown reduced bleeding with FVIII levels as low as 1% to 3% and would be the

ideal population for a later phase gene transfer trial intended to demonstrate efficacy.[36] For current early phase trials such as this study, however, FVIII activity levels in the blood remain the most useful efficacy surrogate.

One possible risk associated with gene transfer is that presentation of a normal transgene product through major histocompatibility complex (MHC) class I mechanisms might lead to the formation of antibodies to the transgene product.[6,31] No FVIII inhibitory activity was demonstrable in this study by either direct measurement or by pharmacokinetics of administered FVIII product. In fact, availability of FVIII product at the end of phase 1 of the study (week 13) was greater than at baseline. This finding may be explained by a saturation of FVIII binding sites[37,38] due to low-level FVIII protein production due to the vector.

There were no adverse effects related to administration of hFVIII(V), other than mild infusion-related symptoms. It is likely that the single, transient, very low-level positive signal in one semen specimen at week 29 in this study was a false-positive result. All preceding and subsequent samples in the subject were negative, and, in rabbit studies there was no evidence for vector transmission to the germ line with hFVIII(V).[11] There appeared to be no adverse effects of hFVIII(V) infusion on the clinical course of HIV or HCV infection.

This was the first human study in which a retroviral vector was administered into a peripheral vein. The benign safety profile seen in this study is consistent with past experience in more than 1000 subjects who have received gene transfer mediated by retroviral vectors with no adverse consequences related to random integration. Nonetheless, the need for continued safety monitoring of these patients is underscored by a recent report to regulatory agencies of a leukemic syndrome developing in a child who had received ex vivo retroviral gene transfer in a French clinical trial.[39] The etiology of this event is being evaluated, and a full published report is not yet available. However, it is believed that the leukemia is linked to the integration of the therapeutic vector near a cellular gene that was then overexpressed. Recently, a second child also experienced a similar adverse event.[40] Although both the French clinical trial and this trial used vectors based on retroviral constructs, there are major differences between the vectors, the methods of delivery, and the subjects. In particular, the French trial used ex vivo transduction of highly enriched hematopoietic stem cells with very high vector to cell ratios, and the subjects were young children with severe immunodeficiency.[41] For the current trial with FVIII(V), a follow-up protocol has been in place for annual visits to the clinic by each subject to provide annual reports on long-term safety to appropriate regulatory agencies. The follow-up protocol is being amended, per recent FDA request, to biannual visits for the first 5 years after vector administration.

Much remains to be learned. As with other human gene transfer trials for hemophilia using different techniques and approaches,[42,43] it appears that animal studies may not accurately predict the doses of vector preparations required to achieve therapeutic expression of FVIII or FIX in humans.

In summary, this phase 1 trial using intravenous infusion of hFVIII(V) demonstrates that hFVIII(V) is safe at the doses and route of administration used, persists in PBMCs as long as one year, is associated with measurable factor VIII concentrations in some individuals, and with increased available plasma factor VIII after infusion of exogenous factor VIII without the development of inhibitors. This excellent safety profile and the potential clinical benefits suggest that further testing of this vector is warranted.

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Patients were enrolled and treated at the following sites: University of California at Davis, Sacramento, CA; University of Pittsburgh, General Clinical Research Center, PA; University of North Carolina, Chapel Hill; and Wayne State University, Detroit, MI.

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